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Chromatin Regulators of Genomic Imprinting

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Abstract

Genomic imprinting is an epigenetic phenomenon in which genes are expressed monoallelically in a parent-of-origin-specific manner. Each chromosome is imprinted with its parental identity. Here we will discuss the nature of this imprinting mark. DNA methylation has a well-established central role in imprinting, and the details of DNA methylation dynamics and the mechanisms that target it to imprinted loci are areas of active investigation. However, there is increasing evidence that DNA methylation is not solely responsible for imprinted expression. At the same time, there is growing appreciation for the contributions of post-translational histone modifications to the regulation of imprinting. The integration of our understanding of these two mechanisms is an important goal for the future of the imprinting field.

Keywords

genomic imprinting; epigenetics; DNA methylation; Polycomb; histone modifications

1. Introduction

Diploid, sexually reproducing organisms inherit two sets of chromosomes, one from each parent, and so possess two copies of each autosomal gene. For the majority of these genes, both alleles are expressed, or not, as appropriate for the cell type. There are, however, genes that are monoallelically expressed in a parent-of-origin specific manner. The mammalian genome contains about 150 genes that are imprinted (a complete up-to-date list can be found at: <http://www.mousebook.org/catalog.php?catalog=imprinting> [1]). As a result, mammalian development requires genetic contributions from both a mother and a father [2, 3].

Imprinted genes are generally found in clusters containing both maternally and paternally expressed genes, although a few singleton imprinted genes exist as well. Each imprinted cluster contains a regulatory element known as an imprinting control region (ICR). By definition, deletion of an ICR results in loss of imprinting (i.e. biallelic silencing or biallelic expression) of all genes within the cluster [4, 5]. Thus, the ICR is the master regulator of imprinting in the region.

Two well-defined mechanisms of imprinted gene regulation have been described: the insulator model and the non-coding RNA (ncRNA) model. In the insulator model (Fig. 1A), the ICR is bound by the insulator protein CTCF binding factor (CTCF) on only one allele.

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The insulator regulates access of genes on either side of it to shared enhancers. On the allele where CTCF is bound, only the genes found between the insulator and the enhancer can be activated; the insulator prevents genes on the far side from accessing the enhancers. When CTCF is not bound, these genes can be activated by the enhancers. See also Matzat and Lei in this issue for a detailed review of recent advances in our understanding of the nature of insulators. The other major imprinting model is the ncRNA model (Fig. 1B). In this case, the ICR is the promoter of a long ncRNA, which is expressed from only one allele. On this allele, expression of the ncRNA silences the rest of the genes in the domain in *cis*. On the other allele, the ncRNA is silenced and the rest of the genes are active.

Imprinted genes span a range of functions, but many are expressed during embryonic development and have roles in the regulation of cell growth and in placental function. Imprinted genes are also involved in maternal behaviors, postnatal energy homeostasis and neurological function [6, 7].

There are a number of rare congenital disorders that are caused by defects in imprinting. For example, failure to express genes within the *SNRPN* imprinted domain results in Prader-Willi Syndrome (PWS) and Angelman Syndrome (AS). Genetic or epigenetic abnormalities in the *H19/IGF2* or *KCNQ1* domains result in Beckwith-Wiedemann Syndrome (BWS) or Silver-Russell Syndrome (SRS), depending on which parental allele is affected [8]. Additionally, recent studies have shown a connection between the use of assisted reproductive technologies, such as *in vitro* fertilization, and imprinting disorders [8].

This review will focus on the nature of the epigenetic imprinting mark and the roles of different chromatin modifications, such as DNA methylation and post-translational modification of histones, in genomic imprinting.

2. The Role of DNA Methylation in Imprinting

2.1 DNA methylation

Imprinted clusters contain differentially methylated regions (DMRs), which are characterized by DNA methylation of only one parental allele. DNA methylation is the covalent attachment of a methyl group to the 5-position carbon on cytosine residues. In mammals, this modification is almost exclusively found on cytosines followed by guanines (CpG sites). It is generally considered to have a repressive effect on transcription, though this is not always the case. DNA methylation is a stable mark, which is propagated through replication by the maintenance DNA methyltransferase, DNMT1 [9, 10]. DNA methylation can also be established by the *de novo* DNA methyltransferases, DNMT3A and DNMT3B, and can be removed by either active or passive demethylation [11, 12]. The mechanisms of demethylation are still under investigation, and will be discussed in further detail below. Importantly, all known ICRs are germline DMRs, meaning that differential methylation is established either during oogenesis or spermatogenesis, and this pattern is maintained after fertilization despite widespread erasure of DNA methylation during preimplantation development [13, 14]. Differential methylation can also be found outside of ICRs, but it is typically established after fertilization [15]. For this reason, DNA methylation is the best candidate for the mark that designates the parental identity of each allele.

2.2 Establishing the methylation imprint in the germline

2.2.1 Erasure of prior imprints—Sex-specific methylation of ICRs is established during gametogenesis (Fig. 2A). Inherited methylation is erased and new methylation is put in place in preparation for inheritance by the next generation. This process begins as primordial germ cells (PGCs) are specified and migrate to the developing gonad. In mice, PGCs travel from the extra-embryonic mesoderm to the genital ridge between embryonic day 7.5 (E7.5) and

E12.5. During this time, they undergo widespread chromatin changes. These changes include loss of both repressive and activating histone modifications, loss of DNA methylation, and reactivation of the silent allele of imprinted genes [16–18]. In humans, the process of germline reprogramming is not as well understood, but PGCs are specified and begin migrating sometime during the first four weeks of gestation, arriving in the fetal gonad between 29 and 33 days post-conception (dpc) [19].

The mechanism by which DNA methylation is lost in PGCs is not completely understood. Demethylation occurs fairly rapidly between E10.5 and E11.5, at a time when most PGCs appear to be in the G2 phase [18]. This would seem to support an active method of demethylation. The recent discovery of the TET dioxygenases, which convert mC to hydroxymethylcytosine, suggests one possible method of demethylation. *Tet1* and *Tet2* are expressed in PGCs [20, 21] and could function in this capacity. Whereas *Tet1*^{-/-} PGCs are reported to undergo normal demethylation [22], defects in a small number of imprinted genes were reported in *Tet1*^{-/-}; *Tet2*^{-/-} double knock out mice, suggesting at least a partial role of hydroxymethylation in DNA demethylation [23]. Base excision repair proteins, such as PARP1, APE1, and XRCC1, have also been implicated in active demethylation during PGC reprogramming [20]. Finally, since PGCs are still undergoing mitosis during the period of demethylation, passive demethylation (where the newly synthesized strand is not methylated during S phase) could be involved. Saitou and colleagues recently showed that PGCs undergo mitosis more rapidly than previously realized, with a doubling time of approximately 12 hours between E9.5 and E12.5 [24]. In addition, they found that PGCs at this time express little to no *Dnmt3a*, *Dnmt3b*, or *Dnmt3L*, and DNMT1 is not localized to replication forks. Taken together, these results support a combination of hydroxymethylation and passive demethylation as the best explanation for loss of methylation at imprinted loci in PGCs. The mechanisms of DNA demethylation will be discussed below in the context of the preimplantation embryo.

2.2.2 Timing of *de novo* methylation—New methylation imprints are subsequently established in females postnatally (Fig. 2B), while in males they are established beginning prenatally (Fig. 2C). Methylation of ICRs is established in the female germline during oocyte growth and is completed by the time oocytes arrest at the metaphase II (MII) stage [14, 25]. Trasler and colleagues [25] analyzed the methylation status of multiple imprinted loci in young female mice, when a large number of follicles mature simultaneously. They found that primary stage oocytes from mice at 1 day post partum (dpp) completely lacked DNA methylation. DNA methylation began to accumulate on maternally methylated DMRs around 10 dpp. The *Peg3* and *Snrpn* DMRs gained methylation first, followed by the *Igf2r* DMR and finally the *Peg1* DMR. The amount of methylation correlated with the size of the oocyte. To determine whether the degree of methylation at maternally methylated ICRs was determined primarily by the age or size of the oocyte, Kono and colleagues collected oocytes from female mice at 10, 15, and 20 dpp, as well as from adults, sorted them by size, and examine methylation of multiple imprinted loci [26]. In adolescent mice, they found that the degree of methylation depended on the size of the oocyte, regardless of age. In adult mice, methylation also proceeded as oocytes grew, but oocytes of a particular size from adults generally were slightly less methylated than oocytes of the same size from adolescent mice.

Interestingly, Trasler and colleagues also found that the maternally inherited copy of the *Snrpn* gene was methylated earlier in the course of oocyte development than the paternally inherited copy [25]. This is very similar to what was observed at the *H19/Igf2* ICR during spermatogenesis, where the paternal allele acquired methylation in mitotically arrested prospermatogonia between E14.5 and E15.5, while the maternal allele only began acquiring methylation at E18.5 and was not completely methylated until after the onset of meiosis,

postnatally [13, 27]. These results indicate that the chromatin at imprinted loci carries some history of its previous methylation status, possibly in the form of other chromatin modifications, despite reprogramming in PGCs.

2.2.3. *De novo* methyltransferases—Methylation is accomplished by the *de novo* methyltransferases, DNMT3A, DNMT3B, and DNMT3L. Evidence points to DNMT3A as the major *de novo* methyltransferase involved in imprinting. Embryos derived from oocytes conditionally deleted for *Dnmt3a* die during embryogenesis and lack methylation of all maternally methylated germline DMRs [28]. Conditional deletion of *Dnmt3a* in the male germline results in sterility. In spermatogonia, prior to failure of spermatogenesis, methylation was absent at two of the three germline DMRs examined [28]. In contrast, DNMT3B is largely uninvolved in methylating imprinted domains. *Dnmt3b*^{-/-} oocytes are fully viable and display normal methylation of all maternal germline DMRs [28]. The methylation status in *Dnmt3b*^{-/-} sperm was likewise unaffected, and null sperm produced viable offspring [28]. Instead, DNMT3B is more important for the methylation of repetitive DNA, such as the centromeric minor satellite repeats [11].

The *de novo* methylation of germline DMRs must be targeted specifically to imprinted loci and needs to be established at different locations in males and females. Multiple mechanisms have been proposed to target *de novo* methylation to the correct loci. DNMT3L is an enzymatically inactive co-factor that stimulates methylation by DNMT3A and DNMT3B [29]. It is required for acquisition of DNA methylation at imprinted loci in gametes [30]. *Dnmt3L*^{-/-} females were viable and produced oocytes, but the oocytes lacked all methylation at maternally methylated ICRs. If *Dnmt3L*^{-/-} oocytes were fertilized, the resulting embryos died *in utero*. The loss of methylation appeared to be specific to imprinted loci, as genome-wide methylation was unaffected [30, 31]. However, more recently, whole-genome bisulfite sequencing of *Dnmt3L*^{-/-} oocytes has revealed that they lack not only methylation of germline DMRs, but also have hypomethylation of intragenic sequences and partial hypomethylation of some retrotransposons [32]. Furthermore, whole-genome bisulfite sequencing has revealed extensive non-CG methylation in oocytes, which is also reduced globally in the absence of DNMT3L function [33]. Loss of DNMT3L function led to sterility in males [30, 31], largely due to the reactivation of parasitic DNA species. DNA methylation was absent from ICRs [34], but repetitive sequences, including Line and Sine elements, IAPs, and minor and major satellites repeats, were also hypomethylated [35]. These results indicate that, although critical for *de novo* methylation of imprinted loci, DNMT3L is involved in methylation of a variety of other sequences as well.

The DNMT3 proteins do not bind specific DNA sequences, so they are most likely targeted via interactions with cofactors. In fact, *in vitro* assays have indicated that DNMT3L can bind histone H3. It does so only when the tail of H3 is unmethylated at lysine 4 [36]. Because methylation of this residue is generally associated with areas of active transcription, it may provide a mechanism for repelling DNMT3L from regions that need to remain unmethylated.

The crystal structure of the DNMT3A/DNMT3L complex also suggested a possible targeting mechanism. These proteins form a tetramer composed of two molecules each of DNMT3A and DNMT3L. In this configuration, the active sites of the two DNMT3A molecules are spaced a set distance apart, equal to about one turn of the DNA double helix, or 8–10 base pairs (bp) [37]. Cheng and colleagues [37] analyzed the sequences of maternally- and paternally-methylated ICRs and found that CpGs spaced 8–10 bp apart are characteristic of maternally methylated ICRs but not paternally methylated ICRs. The recognition of this CpG spacing could target *de novo* methylation to ICRs specifically in the female germline. However, subsequent studies have found that CpGs with this spacing are

not limited to imprinted loci. Thus, CpG spacing alone cannot account for accurate targeting of *de novo* methylation in the female germline [38].

2.2.4 *Trans* factors involved in targeting methylation—Other *trans* factors are likely required to target *de novo* methylation to imprinted loci. In fact, there is some circumstantial evidence that the protein BORIS (a paralog of the key insulator protein CTCF, described above) and the histone methyltransferase PRMT7 may be involved in methylation of the *H19/Igf2* ICR in the male germline [39]. In addition, most cases of familial biparental hydatidiform mole, which results from failure to establish and/or maintain methylation at multiple loci, are caused by mutations in *NLRP7* [40]. Mutations in *C6ORF221* have also been found in patients with this condition [40, 41].

Finally, recent studies from the Kelsey lab have suggested that transcription across ICRs during oocyte development is required for their methylation [42]. At the *Gnas* locus, which is characterized by a variety of overlapping transcripts, transcription of the *Nesp* gene proceeds through the ICR during the time that methylation is established. Truncation of the transcript resulted in failure to establish methylation at the ICR and loss of imprinting throughout the locus. When the study was expanded to other maternally methylated ICRs, the authors reported that many had transcripts crossing the ICRs during oocyte development and methylation establishment, suggesting that transcription may be a general mechanism involved in the establishment of methylation at imprinted loci in the oocyte. In fact, a similar mechanism likely operates in the male germline, as transcription was detected at the *Igf2* ICR and Ig-DMR at the time of imprint establishment [43].

2.3 Changes in DNA methylation in the preimplantation embryo

2.3.1 Mechanisms of DNA demethylation—In the preimplantation embryo, the genome again loses substantial amounts of 5mC, then regains it as differentiation begins [12, 44, 45]. While the identities of the DNA methyltransferases have been established for more than 15 years, the enzyme(s) that remove DNA methylation have remained a mystery, one that is only now beginning to be solved. In theory, DNA methylation could be removed either by an active, enzymatic method, or a passive one. The evidence for active, enzymatic demethylation came from immunohistochemistry studies, where anti-5mC staining was nearly completely lost from the paternal genome before the first cleavage division, too fast to be explained by passive demethylation [12, 44, 45]. Furthermore, loss of 5mC staining of the paternal pronucleus appears to begin prior to S phase [44, 45]. Although many possible mechanisms have been proposed for active demethylation, there was much excitement in 2009 when two groups reported a previously unrecognized base, hydroxymethylcytosine (hmC), and the family of enzymes that synthesize it from mC—TET1, TET2, and TET3 [46, 47]. This base appeared to be limited to a few cell types, specifically embryonic stem cells (ESCs) and Purkinje cells, and was absent from other cell types tested. It was quickly realized that conversion of mC to hmC could represent a mechanism of either active or passive demethylation. hmC could be further enzymatically altered to eventually yield unmodified mC. Alternatively, hmC might prevent maintenance of DNA methylation if it were not recognized by DNMT1. Xu and colleagues provided evidence that TET3 may demethylate the paternal genome in the preimplantation embryo [48]. Mice with a conditional deletion of *Tet3* in the germline appeared normal and healthy but had reduced fertility. They had fewer, smaller litters as well as higher rates of resorption and embryos with abnormalities. When *Tet3*^{-/-} oocytes were fertilized by wild-type sperm, the resulting zygotes lacked hmC on the paternal pronucleus by immunostaining, whereas control zygotes had visible hmC staining of the paternal pronucleus. Moreover, anti-mC staining of the paternal pronucleus was rapidly lost in zygotes conceived with wild-type oocytes, but it remained in zygotes from *Tet3* null oocytes. Bisulfite sequencing also indicated that

methylation levels were higher at repetitive elements such as Line1 elements, as well as critical pluripotency genes such as *Oct4*, in zygotes conceived from *Tet3* null oocytes than from control oocytes. These results suggest that demethylation of the paternal pronucleus in the zygote occurs via conversion of mC to hmC by TET3. Furthermore, this demethylation is important for proper embryonic development. However, the fact that some embryos lacking maternal TET3 were able to develop successfully indicates, surprisingly, that this process is not absolutely necessary for survival. Notably, it is not clear how the hmC is resolved. It has alternatively been proposed that the base is further oxidized [49–51], removed by the repair pathway [49], or lost passively through replication [52].

2.3.2 Maintenance of methylation at imprinted loci—Despite the large-scale demethylation of the genome in preimplantation embryos, imprinted loci must retain their allele-specific methylation. How are they protected from demethylation? The DNA methyltransferase DNMT1 is considered the maintenance methyltransferase because it displays greater enzymatic activity *in vitro* when using hemimethylated DNA (where one of the DNA strands is methylated) as a substrate than when using unmethylated DNA [53]. Additionally, it is expressed broadly in mitotically active cells and localizes to replication forks in S phase [9]. In the absence of DNMT1, DNA methylation would be expected to decrease by half with each round of cell division and so be rapidly lost. It was thought that zygotic transcription of *Dnmt1* did not begin in mouse until around E7 [54, 55]. This could explain the genome-wide loss of methylation in the preimplantation embryo, but not how imprinted genes retain methylation. An oocyte-specific form of DNMT1, DNMT1o, is present in oocytes and preimplantation embryos, which made it an attractive candidate for the maintenance methylation of imprinted loci. In fact, embryos derived from *Dnmt1o*^{-/-} oocytes displayed loss of methylation of imprinted genes, but only by about half [56, 57]. This result was consistent with the observation that DNMT1o was cytoplasmic during preimplantation development except at the 8-cell stage, when it became nuclear. The authors suggested that DNMT1o was required for maintenance methylation, but only during this one round of DNA replication. Subsequent studies have questioned the accuracy of the DNMT1o immunohistochemistry results while demonstrating that low levels of the somatic form of DNMT1, DNMT1s, are in fact present during preimplantation development [58, 59]. The genetic evidence indicates that both isoforms are likely to be involved in preimplantation methylation maintenance. Maternal and zygotic deletion of both isoforms of DNMT1 resulted in complete loss of methylation from all maternal DMRs [58]. Additionally, injection of a transgene expressing *Dnmt1s* could rescue loss of DNMT1o function [60], suggesting that the activity of the two isoforms is the same, and they are only distinguished by the timing of their expression.

Trans-acting factors are expected to be necessary to direct DNA methyltransferase activity specifically to imprinted loci, given that the rest of the genome appears to lose methylation during preimplantation development. A number of these factors have been identified. STELLA (also known as PGC7), a marker of PGCs [61, 62], has a maternal effect on maintenance of DNA methylation. Although methylation is established correctly in *Stella*^{-/-} oocytes, both paternal and maternal DMRs display hypomethylation in embryos derived from these oocytes [63]. STELLA appears to maintain methylation of imprinted loci by protecting mC residues from conversion to hmC [64]. Additionally, ZFP57 is required for methylation of multiple imprinted domains. ZFP57 is a KRAB zinc finger protein, one of a class of proteins that repress transcription by recruiting KAP-1/TIF1β corepressor complexes [65, 66]. ZFP57 is required both maternally and zygotically for establishment and maintenance of methylation at imprinted loci [67]. Zygotic loss-of-function caused reduced methylation at a number of imprinted loci and lethality of some embryos. Loss of function of both maternal and zygotic ZFP57 resulted in embryonic lethality associated with complete loss of methylation from the *Snrpn*, *Peg1*, *Peg3*, *Peg5*, and *Dlk1* DMRs (but not

the *H19* and *Igf2r* DMRs). The role of ZFP57 in imprinting was independently established in studies of human cases of transient neonatal diabetes (TND) [68]. TND is caused by hypomethylation of the promoter of the imprinted gene *PLAGL1* [69]. Mackay et al. [68] determined that multiple cases of TND involved mutations in *ZFP57*. Patients with *ZFP57* mutations had additional clinical features not usually associated with TND but rather with BWS, and also had hypomethylation of multiple imprinted loci. These results indicate that *ZFP57* is required for maintenance of methylation at multiple imprinted loci in both mouse and human. Finally, studies that reduce the dosage of other key proteins in the genome during early development have shown that many factors, not surprisingly, are involved in maintaining genomic imprints. For example, MBD3, a component of the repressive NuRD complex [70], is required in the preimplantation embryo to specifically maintain methylation and repression of the paternal allele of *H19* [71].

2.3.3 High-resolution views of DNA methylation in the early embryo—Our understanding of the methylation dynamics of preimplantation embryos has been largely based on immunohistochemistry and bisulfite sequencing of imprinted loci, repetitive elements, and pluripotency genes. Recently, it has become possible to examine methylation in embryos at the genome-wide scale. Meissner and colleagues [72] used their reduced representation bisulphite sequencing (RRBS) technique, which evaluates about 5% of the genome, to generate methylation maps of the mouse genome in gametes, zygotes, preimplantation embryos, and E6.5–E7.5 epiblasts. Generally, their results supported earlier findings. Methylation levels dropped substantially between sperm and zygotes, continued to decrease during preimplantation development, and then rose again in the post-implantation embryos. What was not well appreciated previously was how much the methylation landscape of the zygote and preimplantation embryos is determined by the oocyte. The vast majority of the methylation changes between gametes and zygotes involved sites that were methylated in sperm, not in oocytes, and lost that methylation after fertilization. Many of these sites were home to retroelements. The authors also identified many regions that inherited methylation from only one gamete and retained this differential methylation until implantation. Paternally methylated regions were more common and tended to be intergenic, while maternally methylated regions were enriched for CpG-rich promoters.

2.4 Loss of imprinting in *Dnmt1* mutants

The importance of methylation in imprinting was definitively shown with the first *Dnmt1* knockout mouse [73]. This deletion resulted in mid-gestation lethality, global loss of methylation, and loss of imprinting [10, 73]. It has since been shown that this initial deletion, now known as *Dnmt1ⁿ*, is only a partial loss-of-function mutation. It was intended to delete part of exon 1 and exon 2, including part of the 5'UTR, the translation start site, and the first 27 codons, replacing them with the neomycin-resistance gene driven by the *Pgk1* promoter. However, three more exons, including the major ATG site, were subsequently found upstream [74]. Thus, *Dnmt1ⁿ* deletes about 60 amino acids (~amino acids 112–172) in a region of somatic DNMT1 involved in interactions between it and PCNA [75] and adjacent to a region required for maintenance of methylation specifically at imprinted loci [76]. Two more *Dnmt1* mutant alleles were subsequently constructed. *Dnmt1^s* contains an insertion in a region important for targeting the protein to the replication fork, and *Dnmt1^c* deletes the PC and ENV motifs, critical portions of the catalytic domain, and is presumed to be a null allele [77]. *Dnmt1^{n/n}* ESCs and embryos both had about a 70% reduction in the amount of cytosine methylation genome-wide, while *Dnmt1^{s/s}* and *Dnmt1^{c/c}* ESCs had a more severe loss of methylation. *Dnmt1^{s/s}* and *Dnmt1^{c/c}* embryos died about a day earlier during development than *Dnmt1^{n/n}* embryos.

These mutations have been used for a number of studies examining the role of DNA methylation in imprinting. From these results, there is little doubt that DNA methylation is critically involved. All *Dnmt1* mutants display perturbations in allele-specific and overall expression levels of imprinted genes. It is notable, however, that loss of imprinting in these mutants is not complete. For example, there is less widespread loss of imprinting in *Dnmt1^{n/n}* embryos than in *Dnmt1^{s/s}* and *Dnmt1^{c/c}* embryos; *Igf2r* is only biallelically expressed in the latter two cases. Furthermore, studies of *Dnmt1* mutants have suggested that there may be tissue- and domain-specific differences in the requirement for DNA methylation. In the *Kcnq1* cluster, four genes that are only imprinted in the placenta (*Osbp15*, *Tssc4*, *Cd81*, and *Ascl2*) remain imprinted in *Dnmt1^{c/c}* placentas [78–81]. Interestingly, the alleles of these genes are differentially marked by histone modifications in the placenta, with repressive marks on the silent allele and active marks on the active allele [80, 82]. These observations have led to the proposal that histone modifications, rather than DNA methylation, are the critical regulators of imprinting in the placenta [80, 82, 83]. Placentas derived from *Dnmt1o^{-/-}* oocytes, however, do have biallelic expression of these placental genes, indicating that they still require DNA methylation early on [81]. Perhaps this DNA methylation directs the deposition of histone modifications, which are then able to maintain proper expression even when the DNA methylation is lost. It was recently shown that some of the genes thought to be maternally expressed in placenta are in fact not expressed in placenta but highly expressed in maternal tissues such as uterus and blood [89]. For example, although *Tssc4* and *Ascl2* were found to be imprinted in the placenta, *Osbp15* was not. Nevertheless, there is evidence that histone modifications are involved in regulating imprinting.

3 The Roles of Histone Modifiers in Imprinting

3.1 The Polycomb proteins

The Polycomb Group (PcG) proteins confer repressive functions that play important roles in stable, heritable gene repression, particularly in euchromatic regions [84]. PcG proteins, for example, silence the *Hox* loci, explaining their homeotic-like mutant phenotypes [84]. PcG proteins operate as large multi-protein complexes, including Polycomb Repressive Complex 1 (PRC1) and PRC2 (Fig. 3), though the complete number of complexes, or the number of varieties of each complex, is still being debated [84]. Both complexes covalently modify histones, which is likely part of the mechanism by which they repress transcription [84]. In particular, PRC1 monoubiquitinates histone H2A on lysine 9 (H2AK119), and PRC2 methylates histone H3K27 [84].

Mammalian PRC1 is a large complex containing RING1A/RNF2, BMI-1, PHC1–3, CBX, and a number of other proteins. CBX2, CBX4, and CBX6–8, the orthologs of the *Drosophila* protein Polycomb (Pc), contain a chromodomain that can bind to H3K27me3. RING1A and RNF2 are E3 ubiquitin ligases responsible for catalyzing H2AK119ub1 [85]. PRC2 consists of three major protein players, Enhancer of Zeste 2 (EZH2), Embryonic Ectoderm Development (EED), and Suppressor of Zeste 12 (SUZ12), as well as two binding proteins, RbAp46 and RbAp48, and a variable array of interacting factors. PRC2 has been shown to interact with both DNA methyltransferases and histone deacetylases under certain circumstances [86, 87]. EZH2 (also referred to as KMT6) is the enzymatically active histone methyltransferase member of the complex [88].

H3K27me3 is widely distributed throughout the genome, covering discrete areas and larger swaths of sequence [89–91]. Regions of H3K27me3 are negatively correlated with RNA Polymerase II (PolII) occupancy, supporting the repressive role of this modification. The PRC2 proteins themselves are often found bound to the same loci as the modification. In *Drosophila*, PRC2 binds to specific sites called Polycomb Response Elements (PREs). It is

less clear whether equivalent sequences exist in vertebrates. In mouse ESCs, PcG proteins and H3K27me3 were found preferentially at genes encoding transcription factors, especially those with critical roles in development and differentiation, which led to the idea that PcG proteins maintain pluripotency by preventing expression of genes that induce differentiation [89]. A number of these developmental regulators were also found to be enriched for the activating mark H3K4me3 in ESCs [92]. It is thought that these “bivalent” domains represent a means to keep the promoters of critical differentiation regulators in a poised state, so that they can quickly resolve to either a silent or activated state upon differentiation [92].

3.1.1 Polycomb proteins and imprinting—With roles in stable repression of facultative heterochromatin, PcG proteins are good candidates for a role in repression of the silent allele of imprinted genes. Both the protein complex PRC2 and its methylation mark, H3K27me3, have been observed at imprinted loci in ESCs and in embryonic and extra-embryonic tissues. Their presence is limited to a subset of imprinted domains.

Umlauf et al. [82] observed allele-specific H3K27me3 throughout the *Kcnq1* domain in ESCs. It was enriched on the silent maternal allele of the ncRNA-encoding *Kcnq1ot1* locus and on the silent paternal allele of other genes in the domain. The same pattern of histone modifications was also observed in embryos and placentas, though the allele-specific modifications were found more broadly in ESCs and placenta than in the embryo, where it was limited to some promoter regions. The PRC2 complex itself was also observed on the paternal allele of *Ascl2* and *Cdkn1c*. Lewis et al. [80] likewise observed a slight enrichment of H3K27me3 on the silent paternal allele of genes in the *Kcnq1* domain in placenta, which was dependent on the presence of the ICR.

Verona et al. extensively analyzed histone modifications at multiple imprinted domains in ESCs and mouse embryonic fibroblasts (MEFs) [93]. They found that H3K27me3 was present on the methylated allele of the ICR in the *Kcnq1* and *Snrpn* domains in ESCs but not in MEFs. This was not true of all imprinted domains, however, as this modification was not present in an allele-specific manner at any of the regions analyzed in the *H19/Igf2* domain, including the ICR and the *H19* promoter and coding region. In contrast, Han et al. [94] observed allele-specific H3K27me3 in MEFs at multiple sites in the *H19/Igf2* domain, including the paternal allele of the *H19* gene and the maternal allele of the *Igf2* gene. However, they also found no difference in H3K27me3 levels on the two alleles of the *H19/Igf2* ICR. Based on their localization, it seems likely that PRC2 and H3K27me3 are involved in imprinted gene expression at some level. A few studies have begun to establish which genes they regulate, and in which tissues.

3.1.2 Loss of imprinting in *Eed* mutants—Magnuson and colleagues examined imprinting in *Eed*^{-/-} embryos [95]. The *Eed* mutation was isolated in an ENU screen [96]; mutant embryos had severe developmental defects, including anterior-posterior pattern defects, and died before E9.5 [97, 98]. By analyzing expression of imprinted genes between E5.5 and E8.5, they found that a subset of imprinted genes was biallelically expressed, specifically *Ascl2*, *Grb10*, *Cdkn1c*, and *Gtl2* [95]. The authors performed bisulfite sequencing on the DMRs associated with these genes. Surprisingly, they did not see large-scale changes in DNA methylation at any of these loci. Instead, they noted changes at individual CpGs and argued that these changes might explain the altered expression. Alternatively, loss of repression might have been independent of DNA methylation.

Another surprising aspect of these results is the set of genes identified. *Cdkn1c* and *Ascl2* are found in the same imprinted domain, but *Grb10* and *Gtl2* are each found in a different domain. It is unclear what these particular genes have in common, or why they would share

a mechanism of repression that is not utilized by their neighbors. One possibility is that EED actually has a more important role in repression of imprinted genes than was revealed by this study. Members of the PRC2 complex, including EED, are present at high levels in the oocyte [99, 100], meaning that PRC2 was present in the oocyte from which the *Eed*^{-/-} embryos were derived during imprint establishment, and maternal PRC2 may have persisted well past fertilization despite the absence of functional zygotc EED.

3.1.3 Polycomb proteins and genomic contraction—Terranova et al. [101] reported that PRC2 interacts with the ncRNA *Kcnq1ot1* to repress the paternal allele of genes in the *Kcnq1* domain. As described above, the paternally expressed ncRNA *Kcnq1ot1* represses expression of the other genes in the domain in *cis*, which are all maternally expressed. Deletion of the ICR (the *KvDMR1*) [5] or truncation of the ncRNA with a premature transcriptional stop site [102] both result in biallelic expression of genes throughout the locus. It has been unclear, however, whether the ncRNA itself or the process of transcribing it is necessary for repression of the rest of the domain. In this study, the authors showed that *Kcnq1ot1* RNA is localized to a discrete region of the nucleus, overlapping one of the alleles of the *Kcnq1* domain (presumably the paternal allele). It also overlaps an accumulation of PRC1 and PRC2 proteins, as well as H3K27me3 and H2AK119ub1. Additionally, the authors assessed the spatial arrangement of the DNA using two DNA fluorescent *in situ* hybridization probes, one at either end of the domain. They measured the distance between the two probes to give a read-out of how compressed or condensed the region was. The allele associated with *Kcnq1ot1* and PRC2 was much more condensed than the other allele. Although only correlative, these data are consistent with the idea that the ncRNA *Kcnq1ot1* is physically involved in silencing the paternal allele and does so at least in part by associating with PcG proteins and condensing or compacting chromatin. To begin to address mechanisms, the authors examined embryos with mutations in the PRC2 component *Ezh2* and the PRC1 component *Rnf2* and found that both of these proteins were required independently for genomic contraction of the paternal allele. See also Nakagawa and Kagiwada in this issue for a detailed review on the roles of nuclear long ncRNAs in epigenetic regulation.

3.2 H3K9 methylation

Repressive methylation of H3K9 is widely associated with the silent allele of imprinted genes [80, 82, 93, 103]. Thus, it is likely involved in repression of this allele at some level. There is conflicting evidence regarding a role for this modification in the establishment of methylation in the germline. Feil and colleagues noted that H3K9me3 was depleted from ICRs in PGCs prior to *de novo* methylation [43]. In contrast, Nakamura et al. [64] found that STELLA, which binds preferentially to H3K9me2 *in vitro*, is required to protect the maternal genome from demethylation in the preimplantation embryo. The identity of the histone methyltransferases involved in H3K9 methylation at imprinted loci has been more difficult to pinpoint. There are multiple enzymes with this catalytic activity in mammals: ESET, G9A, GLP, PRDM2, SUV39H1, and SUV39H2. G9A is implicated in regulation of certain imprinted genes. *G9a*^{-/-} ESCs lack DNA methylation of the *Snprn* DMR and have biallelic expression of *Snprn*. *G9a*^{-/-} embryos did not display any altered expression of imprinted genes, but some placentally-imprinted genes, including *Osbpl5*, *Cd81*, and *Ascl2*, lost imprinting [83]. In addition, G9A was shown to be required for imprinting of the gene *Tfpi2* [103]. It was also found to interact with the lncRNA *Airn* and the promoter of the imprinted gene *Slc22a3*. Loss of G9A function caused this association to disappear and resulted in LOI of *Slc22a3* [104]. In contrast, SUV39H appears to be uninvolved in methylation of H3K9 at ICRs [105].

4. Conclusions

Genomic imprinting is a classic example of an epigenetic phenomenon, where information about the parental identity of the chromosome is inherited through the germline. The central role of DNA methylation in this process is well established, while recent research has highlighted the fact that DNA methylation is not solely responsible for regulation of imprinting. Our understanding of the roles histone-modifying enzymes play in imprinting regulation has grown, but important questions still remain. How many of these enzymes and modifications are involved, and which ones are important at each imprinted locus? How critical are they to regulation of these loci? Do they control the allele-specific pattern of DNA methylation, affect transcription downstream of DNA methylation, or provide a read-out of transcription only? How much cross-talk is there between DNA methylation and histone modifications at imprinted loci? How do they combine to ensure the proper expression of these critical genes? We look forward to learning the answers to these and other questions in the near future.

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Highlights

- Imprinted genes are regulated by epigenetic mechanisms.
- DNA methylation has an established central role in the regulation of imprinting.
- Some imprinted genes are regulated independently of DNA methylation.
- Histone modifying enzymes also regulate specific imprinted genes.
- Interactions between the two mechanisms are under investigation.

Insulator Model of Imprinting: The *Igf2* Domain

ncRNA Model of Imprinting: The *Kcnq1* Domain

Figure 1.

Mechanism of imprinting. (A) The insulator model is exemplified by the *H19/Igf2* domain. The intergenic ICR is paternally methylated. On the unmethylated maternal allele, CTCF binding prevents enhancers from interacting with the *Igf2* promoter. Instead, the enhancers activate *H19* expression. On the paternal allele, methylation of the ICR spreads to the *H19* promoter, silencing its expression, and prevents CTCF from binding the ICR, allowing the enhancers to activate *Igf2* expression. (B) The ncRNA model is illustrated by the *Kcnq1* domain. The ICR contains the promoter of the ncRNA *Kcnq1ot1*. On the paternal allele, the ICR is unmethylated, allowing *Kcnq1ot1* expression. *Kcnq1ot1* expression silences the paternal allele of the rest of the imprinted genes in *cis*. On the maternal allele, *Kcnq1ot1* is not expressed due to methylation of the ICR, and the adjacent imprinted genes are expressed.

Figure 2. Methylation dynamics in the germline and the embryo. (A) Methylation of imprinted genes versus the genome as a whole during gametogenesis and after fertilization. (B) Imprint establishment in the female germline. (C) Imprint establishment in the male germline.

Figure 3.
The Polycomb Group complexes post-translationally modify histones and repress transcription. The two major Polycomb Group complexes in mammals are PRC1 and PRC2. The core components of each are diagrammed here, as well as the post-translational histone modifications they catalyze.