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Review

Genomic imprinting mechanisms in mammals

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ABSTRACT

Genomic imprinting is a form of epigenetic gene regulation that results in expression from a single allele in a parent-of-origin-dependent manner. This form of monoallelic expression affects a small but growing number of genes and is essential to normal mammalian development. Despite extensive studies and some major breakthroughs regarding this intriguing phenomenon, we have not yet fully characterized the underlying molecular mechanisms of genomic imprinting. This is in part due to the complexity of the system in that the epigenetic markings required for proper imprinting must be established in the germline, maintained throughout development, and then erased before being re-established in the next generation's germline. Furthermore, imprinted gene expression is often tissue or stage-specific. It has also become clear that while imprinted loci across the genome seem to rely consistently on epigenetic markings of DNA methylation and/or histone modifications to discern parental alleles, the regulatory activities underlying these markings vary among loci. Here, we discuss different modes of imprinting regulation in mammals and how perturbations of these systems result in human disease. We focus on the mechanism of genomic imprinting mediated by insulators as is present at the *H19/Igf2* locus, and by non-coding RNA present at the *Igf2r* and *Kcnq1* loci. In addition to imprinting mechanisms at autosomal loci, what is known about imprinted X-chromosome inactivation and how it compares to autosomal imprinting is also discussed. Overall, this review summarizes many years of imprinting research, while pointing out exciting new discoveries that further elucidate the mechanism of genomic imprinting, and speculating on areas that require further investigation.

Contents

1. Introduction

The mammalian genome contains a small number of genes that are subject to genomic imprinting [1,2]. These genes are epigenetically marked with their parental origin such that a given parental allele is expressed while the other is repressed. While the precise nature of the initial epigenetic imprint is currently a matter of intense investigation, it is assumed that the

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parental imprint is set in the germline, as this is the time when the genomes are in distinct compartments and can be differentially modified. After fertilization, the parental imprints must survive the reprogramming that takes place in the preimplantation embryo, including DNA demethylation, protamine-histone exchange and changes in histone modifications [3]. It is worth noting that although a number of imprinted genes remain imprinted throughout the life of the organism, many genes are imprinted in a tissue or temporal-specific way. Finally, while it is hypothesized that the predominant role of these unusual genes is during fetal and placental growth and development [4], postnatal roles for some of these imprinted genes are beginning to be appreciated [5].

Approximately 90 genes have been described as being imprinted, although it is unlikely that we have identified all imprinted genes (for a complete list, see http://igc.otago.ac.nz/home.html and http://www.har.mrc.ac.uk/research/genomic_imprinting/maps.html). One of the hallmarks of imprinted genes is that many are found in clusters throughout the genome [2]. These clusters contain two or more imprinted genes over a region that can span 1 Mb or more. The genes in the clusters, which can be either maternally or paternally expressed, are jointly regulated through an imprinting control region (ICR). The ICRs exhibit parental-specific epigenetic modifications (DNA methylation and histone modifications) that govern their activity. Importantly, deletion of ICRs typically leads to the loss of imprinting of multiple genes within the cluster (for example see [6–10]). Intriguingly, ICRs can be roughly divided into two categories, those that function as insulators and those that serve as a promoter for a regulatory non-coding RNA (ncRNA). Here we will describe clusters that fall into these two major categories and compare autosomal imprinted clusters to imprinted X-chromosome inactivation.

2. Insulator model of imprinting

2.1. H19/Igf2 imprinting

The best-characterized cluster that follows a strict insulator model for imprinted expression is the cluster containing maternally expressed *H19* and paternally expressed *insulin-like growth factor 2* (*Igf2*) [11,12]. This cluster resides at 11p15.5 in humans and is found in conserved synteny on distal chromosome 7 in mice. While most studies on *H19* and *Igf2* have been performed in the mouse, many attributes of these genes including their expression profile and regulatory mechanisms are similar in humans. Remarkably Reik and colleagues have recently shown that the cluster and imprinting are conserved in marsupials, making this imprinting mechanism the most ancient identified to date [13]. In both mouse and human, *H19* and *Igf2* are widely expressed during embryonic development and postnatally downregulated in most tissues. *H19* encodes a fully processed 2.3 kb non-coding RNA and was initially implicated as a tumor suppressor [14,15]. However, it has also been shown to have oncogenic properties [16–20]. *Igf2* encodes a protein that plays a major role in promoting embryonic and placental growth and development [21]. Both *H19* and *Igf2* have recently been shown to encode microRNAs of unknown function [22,23].

As with all imprinted clusters, imprinted expression of *H19* and *IGF2* is regulated by an ICR [designated imprinting center 1 (IC1) in humans and ICR or differentially methylated domain (DMD) in mouse] located between the two genes [24,25]. This region is approximately 5 kb and 2 kb long in humans and mice, respectively (Fig. 1). It acts by regulating interactions between the *H19* and *IGF2* promoters and their shared enhancers, which lie downstream of *H19* (Fig. 1). Deletion of the ICR/DMD results in loss of imprinting (LOI) at *H19* and *Igf2* [8]. Proper imprinting of *H19* and *Igf2* requires that the ICR/DMD is methylated on the paternal allele and unmethylated on the maternal allele. Mutations in CpGs at the ICR/DMD result in hypomethylation and subsequent biallelic expression of *H19* and downregulation of *Igf2* [26].

An important breakthrough in determining the imprinting mechanism at this locus came from the characterization of conserved sequences at the mouse and human ICRs [27,28]. When unmethylated, these sequences bind to the insulator protein CCCTC-binding factor (CTCF) [29–33] (Fig. 1A). CTCF was initially shown to mediate insulator or enhancer blocking activity at similar sequences in the β-*globin* locus [34]. The current model of imprinting regulation at the *H19/Igf2* locus is that binding of CTCF to the unmethylated maternal ICR/DMD protects it from *de novo* methylation and prevents downstream enhancers from activating *Igf2*, leaving them available to activate transcription at *H19* (Fig. 1A). CTCF is unable to bind the methylated paternal ICR/DMD resulting in expression of *Igf2* while *H19* is silenced. Targeted mutation of CTCF sites demonstrated that these sites are necessary for imprint maintenance but not establishment [35–38].

Although it is now well established that the ICR/DMD acts as a CTCF-dependent insulator/enhancer blocker, the mechanism of insulation remains incomplete. Chromosome conformation capture (3C) experiments in mice, which assay for physical interactions between chromosomal regions, have suggested that chromosomal looping is involved in the imprinting mechanism, although the precise nature and function of the looping is debated [39–43]. While there is some consensus that the shared enhancers physically interact with the *Igf2* promoters on the paternal chromosome, interactions on the maternal chromosome remain unclear. Kurukuti et al. report that maternal-specific silencing of *Igf2* results when the ICR/DMD interacts with a matrix attachment region and a differentially methylated region at the *Igf2* locus to generate a tight loop around the *Igf2* gene, thereby physically impeding *Igf2* expression [40]. In contrast, Yoon and colleagues demonstrate that the ICR/DMD forms a transcriptionally unproductive association with enhancers and the inactive *Igf2* promoters on the maternal chromosome, which leads to silencing of *Igf2* [43]. It is unclear how this "decoy" type of model would allow for the interaction between the enhancers and the *H19* promoter, which are necessary for maternal *H19* expression. More recently, we have shown that on the maternal allele, the enhancers make contacts throughout the *H19* coding unit and promoter up to, but not beyond, the ICR/DMD [39]. When the ICR/DMD or CTCF binding sites are deleted on the maternal allele, the enhancers interact throughout the locus, suggesting that the enhancers track along the chromosome until they find a suitable promoter sequence and then the insulator blocks further tracking. There are several reasons why these 3C experiments produce differing results and models. First, the assays were designed to test different interactions (enhancers versus ICR/DMD interactions). Second, different restriction enzymes were used to digest the DNA, which may not have separated regulatory sequences adequately. Third, cells and tissues that were analyzed varied. Finally, the assays were not performed across the entire *H19*/*Igf2* domain in all of the studies and could not be considered highly quantitative. Thus, it is difficult to discern the correctness of one model versus another. Nevertheless, the examination of multiple ICR/DMD mutant alleles demonstrates that long-range allele-specific interactions at the *H19*/*Igf2* locus are dependent upon the ICR/DMD.

Regardless of the precise mechanism of insulator activity, it is clear that distal elements interact. Consistent with the notion of these interactions, recent studies have demonstrated that cohesins, which physically connect sister chromatids, colocalize with CTCF [44,45]. In particular, two cohesin proteins, RAD21 and SMC1, associate with CTCF sites at the *H19/Igf2* DMD in an allele-specific

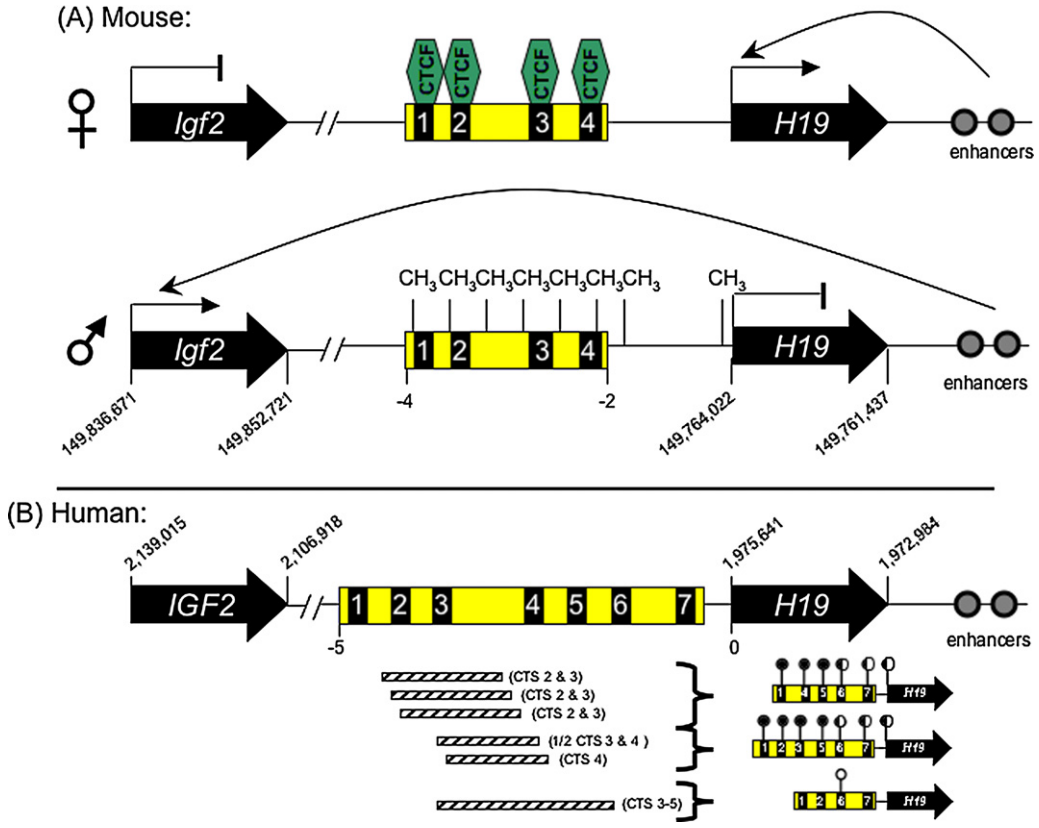

Fig. 1. Regulation of imprinted expression at the human and mouse *H19/Igf2* loci. Shown is the organization of the mouse (A) and human (B) locus (not drawn to scale). Genomic positions in base pairs based on NCBI build 36 (humans) and 37 (mouse). CTCF target sites (designated as CTSs in human and shown with black bars) are indicated within the ICR/DMD/IC1 (yellow bar). (A) Methylation status and gene expression are shown for the mouse *H19/Igf2* locus. Arrows at genes denote active status while arrows pointing right to left denote interactions between enhancers and gene promoters. (B) Microdeletions at IC1 in BWS patients are denoted by hatched-marked bars (modified from [133]). See text for references. The CTSs that are deleted in each case are represented in parenthesis beside each deletion. The corresponding methylation status of the maternally inherited allele for each deletion is depicted to the right as unmethylated (open lollipops), methylated (filled lollipops) or partially methylated (half-filled lollipops).

manner that is comparable to CTCF binding [46], however it remains unknown whether cohesin binding is required for CTCF association or *vice versa*. Further studies to determine the timing of binding of cohesin or additional factors at the ICR/DMD as it relates to imprint establishment in the germline and maintenance throughout development will provide important clues in elucidating the molecular mechanism of *H19*/*Igf2* imprinting.

2.2. H19/Igf2 and disease

LOI and/or aberrant expression of *H19* and *IGF2* is associated with somatic overgrowth and embryonal tumors and has been linked to more than 20 cancer types in human including Wilms' tumor and colorectal, lung, breast and prostate cancers [47]. In addition, the overgrowth disorder Beckwith-Weidemann Syndrome (BWS) and the dwarfism syndrome Silver-Russell Syndrome (SRS) are strongly associated with defects in *H19/IGF2* imprinting [48,49]. Although the precise physiological role of *H19* and *IGF2* in the development of these diseases is unclear, we are starting to appreciate how epigenetic perturbations of the locus can lead to LOI. Analysis of BWS patients revealed that hypermethylation of the IC1, leading to LOI at *H19* and *IGF2*, can result from either maternally inherited deletions in the IC1 or post-zygotically acquired sporadic epimutations that are IC1 sequence-independent and present mosaically [50]. Likewise, analysis of SRS patients revealed that hypomethylation of IC1 can arise independently of changes in the IC1 sequence [51].

In a recent human study, 1.4 kb to 1.8 kb microdeletions at the *H19*/*IGF2* ICR were shown to result in *H19*/*IGF2* LOI and were associated with BWS [52,53] (Fig. 1B). However, in a separate study, a 2.2 kb IC1 deletion was insufficient to cause BWS [54,55] (Fig. 1B). This discrepancy was proposed to result from a difference in spacing/pattern of remaining CTCF sites. The shorter mutations deleted fewer sites resulting in abnormally long clusters of CTCF sites (and CpGs), all of which became aberrantly methylated on the maternal allele. On the other hand, the larger deletion resulted in a cluster of CTCF sites similar to one of the two clusters present on the normal allele. Therefore, the bipartite spacing/pattern of CTCF sites may play a role in determining the epigenetic status of the ICR. Alternatively, the two types of deletions may result in different perturbations of additional regulatory elements such as transcripts that have been described at the ICR/DMD in mice [56] or nucleosome positioning sites (NPSs) present in the intervening sequence that have been proposed to regulate CTCF site availability [57]. Needless to say, further scrutiny of the *H19/Igf2* ICR/DMD is necessary to definitively characterize its regulatory function in genomic imprinting.

3. Non-coding RNA model of imprinting

3.1. Overview

The majority of imprinted loci appear to use a second, ncRNA mechanism of regulation of imprinting in clusters (Fig. 2). The first,

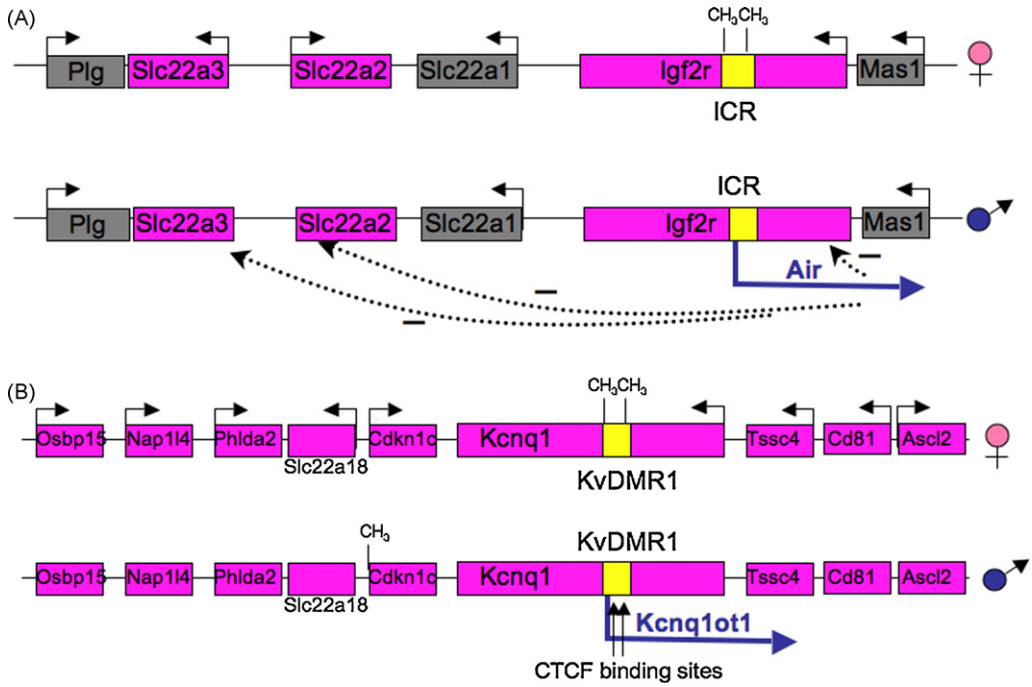

Fig. 2. Regulation of imprinting clusters through long ncRNAs. (A) Imprinting on proximal mouse chromosome 17. *Igf2r*, *Slc22a2* and *Slc22a3* are expressed from the maternal chromosome (pink boxes) and *Air* is expressed from the paternal chromosome (blue arrow). Non-imprinted genes at this domain include *Mas1*, *Plg* and *Slc22a1* (grey boxes). The ICR, which serves as the promoter to *Air*, is shown with a yellow box. The ICR is hypermethylated on the maternal strand, preventing transcription of *Air* and allowing *Igf2r*, *Slc22a2* and *Slc22a3* to be transcribed. On the paternal chromosome the ICR is unmethylated, *Air* is expressed and surrounding genes (*Igf2r*, *Slc22a2* and *Slc22a3*) are repressed (indicated with a dotted arrow). (B) Imprinting of the mouse *Kcnq1* domain. Maternally expressed genes are indicated by pink boxes and the paternally expressed long ncRNA Kcnq1ot1 is shown with a blue arrow. KvDMR1, which is the ICR for this region and harbors the promoter for *Kcnq1ot1*, is designated by a yellow box and is methylated on the maternal allele. The promoter for *Cdkn1c* is methylated on the paternal allele after fertilization. The two CTCF binding sites within KvDMR1 are designated with vertical arrows. See text for additional details. Transcriptional activity of a given gene is indicated by arrows. Not drawn to scale.

and perhaps the best described, cluster in this class is the *Igf2r* cluster, which resides on mouse chromosome 17A [58] (Fig. 2A). *Igf2r* and two neighboring genes, *Slc22a2* and *Slc22a3* (solute carrier 22a2 and 22a3), are expressed maternally. This region also harbors one paternally expressed transcript, Air (antisense Igf2r RNA) that overlaps *Igf2r* and whose expression is critical to the silencing of the maternally expressed genes in *cis* [59]. Furthermore, similar to other imprinted domains, Air expression is regulated by an ICR with parental-specific epigenetic modifications [60]. However, in contrast to other imprinted loci, the genomic organization and the imprinting pattern of the *Igf2r* cluster are only partially conserved in the human syntenic region, on chromosome 6q26–27.

Other loci that express long ncRNAs that are likely to be involved in the imprinting process include the *Gnas* locus [61], the *Dlk1*/*Gtl2* locus [62] and the *Snrpn* locus [2]. The reader is referred to the excellent reviews for more details of the above loci. Here, we will focus on the *Kcnq1* locus. Although the *Kcnq1* locus is immediately adjacent to the *H19*/*Igf2* locus in both mouse and human, it is independently regulated. Importantly, much of what we know about the *Kcnq1* locus is conserved in human [63]. In addition, over half of the documented BWS cases are associated with defects in imprinted gene expression in the *KCNQ1* domain. The majority of these BWS cases exhibit a loss of maternal-specific DNA methylation of the ICR, designated KvDMR1, and subsequent loss of expression of the maternally expressed genes. Also, mutations in the maternally expressed cell-cycle regulator cyclin-dependent kinase inhibitor, *CDKN1C*, constitute about 10% of BWS cases [64].

3.2. ncRNA and the Kcnq1 locus

The *Kcnq1* locus contains one paternally expressed gene encoding a long (>60 kb) ncRNA, *Kcnq1ot1*, and at least eight maternally expressed protein-coding genes, including *Cdkn1c*, *Mash2*, *Phlda2* [63] (Fig. 2B). The locus (and the ncRNA, in particular) is governed by the maternally methylated ICR, KvDMR1, which is located within an intron of the *Kcnq1* gene [65–67]. The promoter for the *Kcnq1ot1* gene resides within KvDMR1. Hypomethylation of the promoter on the paternal allele is associated with *Kcnq1ot1* expression and repression of the adjacent protein-coding imprinted genes, whereas hypermethylation of KvDMR1 on the maternal allele is associated with repression of the ncRNA and activation of the adjacent imprinted genes. Deletion of KvDMR1 on the paternal allele results in a failure to express *Kcnq1ot1* and in biallelic expression of the genes that are normally only expressed on the maternal allele [6,68], suggesting that transcription of the ncRNA Kcnq1ot1 is essential to silence the 8 protein coding genes in *cis*. Furthermore, insertion of a transcriptional stop signal downstream of the promoter on the paternal allele results in the activation of the normally silenced genes on that allele [68]. Thus, similar to what has been reported for the *Igf2r*/*Air* locus, transcription of *Kcnq1ot1* or the transcript itself is required for bidirectional repression of genes in *cis*.

More recently, a new *Kcnq1ot* truncation allele reported by Higgins and colleagues confirmed that absence of the full length Kcnq1ot transcript resulted in loss of imprinting of the linked genes, with one notable exception. In the embryo proper, *Cdkn1c* was still imprinted in a subset of tissues [69]. This study demonstrated that *Cdkn1c* imprinting can be regulated by a mechanism independent of the Kcnqt1ot1 ncRNA. Given that deletion of KvDMR1 results in loss of imprinting of *Cdkn1c* in all tissues [6], the ncRNA-independent mechanism may rely on element(s) within KvDMR1. As such, two CTCF binding sites within KvDMR1 have been identified that are occupied *in vivo* only on the unmethylated paternal allele [70]. Thus, it is possible that for a gene as important to the

growth of the embryo as *Cdkn1c*, redundant mechanisms are in place to assure its appropriate imprinting and consequently, its dosage.

Although a role for long ncRNAs appears certain at autosomal imprinted loci, as well as in X-chromosome inactivation (see below), it is still unclear how these RNAs silence overlapping and nonoverlapping genes that are located in *cis*. Additionally, while some of these silenced genes are several hundred kilobase pairs away, in some loci, such as *Igf2r*, genes more proximal escape the silencing. Multiple models have been proposed to address this complex regulation [2,71]. First, an RNAi based model is suggested in which the long antisense ncRNA forms a double-stranded RNA intermediate complementary to the silenced gene, which then triggers silencing by one of the strategies documented in other systems, including RNA degradation, translational repression or heterochromatin formation [72]. The major problem with this model, however, is that it cannot explain silencing of nonoverlapping genes with no sequence similarity, nor can it account for silencing in *cis*. A second possible mechanism invokes an *Xist* type model of silencing, in which the Xist RNA coats the inactive X-chromosome (see below). The silencing in this case would be much more limited (that is, not coating an entire chromosome), and although coating of the imprinted silenced domains by ncRNA has not yet been reported, such a mechanism cannot be discounted. Finally, models invoking silencing by transcription through the locus have been proposed. In this case, it is the transcription rather than the product of transcription that is important for silencing. Here, transcription could interfere with activators or activate repressors. This particular class of models requires the identity of *cis*-acting regulatory elements, which remain unknown for most imprinted domains.

4. Imprinted X inactivation

An additional type of imprinting is found on the sex chromosomes in the form of dosage compensation. In mammals, dosage compensation of the X-chromosomes between females (XX) and males (XY) is achieved through X-chromosome inactivation (XCI), a process by which one of the two X-chromosomes in females is transcriptionally silenced and the inactive state is clonally transmitted throughout cell divisions [73]. In the postimplantation embryo, either the maternal or the paternal X-chromosome is inactivated, which is referred to as random XCI [74], whereas in the preimplantation embryo and in the extraembryonic lineages, XCI is imprinted with only the paternal X-chromosome (Xp) being silenced [75–79]. Random and imprinted XCI are controlled by a region of the X-chromosome, designated the X-chromosome inactivation center (XIC). The most notable components of the XIC are the *Xist* and *Tsix* genes, which encode long ncRNAs [80]. High expression of *Xist* is generally associated with *cis*-inactivation, whereas *Tsix* is only expressed from the active X-chromosome. Thus, the strategy for using long ncRNA to silence genes in *cis*, as observed for a subset of autosomal imprinted loci, is also invoked here. However, as described below *Xist* coats the chromosome that it inactivates, a process that has not yet been observed at autosomal imprinted loci.

In mice, *Xist* coating is first observed at the two-cell or four-cell embryonic stages [75,77]. Other epigenetic marks are progressively recruited on the Xp between the two-cell and the blastocyst stage, which subsequently leads to X-linked genes becoming silenced in *cis* [75–77,81,82] (Fig. 3). Random XCI occurs after reactivation of the Xp in the inner cell mass of the blastocyst, whereas the inactive Xp is maintained in the trophoblastic and the primitive endoderm cell lineages [74]. The correlation between cell-specific marks on the inactive X-chromosome, as well as their sequential acquisition and loss during development, suggest that yet unknown connections exist between cell lineage determination and XCI.

A maternal imprint, protecting the maternal X-chromosome (Xm) from XCI, is acquired during oocyte maturation [83–85]. The paternal imprint, prompting the Xp to inactivation, is apparently weaker [86,87]. It is regulated by the XIC, as revealed by the ability of an autosomal transgene of the XIC to recapitulate imprinted *cis*-inactivation when it is paternally, but not maternally, inherited [88]. However, the mechanism responsible for the imprint in XCI remains to be elucidated.

The requirement of the *Xist* RNA for the initiation of random XCI and for imprinted XCI in the extraembryonic lineages is well documented [89,90], but has yet to be demonstrated for the establishment of imprinted XCI in the preimplantation embryo. In addition to *Xist*, maintenance of the inactive state of the Xp requires the Polycomb protein, EED, likely through the trimethylation of H3K27, in the trophoblastic but not in the primitive endoderm lineage [91,92]. DNA methylation at the 5′ end of genes is important for the stability of random XCI in the embryo proper, but the methylation pattern of the genes and the requirement for DNA methylation are less clear in extraembryonic tissues [74]. Inactivation of the Xp is not affected in the visceral endoderm of mice lacking the DNA methyltransferase DNMT1 [93] but seems to be altered in trophoblast cells mutated for the newly characterized *SmcHD1* (structural maintenance of chromosomes hinge domain containing 1) gene [94]. Together, these results suggest that imprinted XCI does not rely on DNA methylation in the same manner as autosomal imprinted genes.

Tsix represses *Xist* expression in several cell types [80] and its continuous expression on the maternal X chromosome (Xm) is required in extraembryonic lineages to prevent *cis*-expression of *Xist* and *cis*-inactivation [95,96]. However, it is unlikely that *Tsix* propagates the maternal imprint in the preimplantation embryo, because its expression is detected only in a minority of cells before the blastocyst stage [97]. *Tsix* major promoter lies close to the *DXPas34* 34mer repeat, a regulator of *Tsix* expression [97–102], features of which are reminiscent of ICRs: differential methylation between male and female, though only after implantation [103], binding of CTCF and Yin Yang 1 (YY1) [104-106], and initiation of bidirectional transcription [99]. Differential methylation between sperm and oocyte is not found at *DXPas34* but is present at a few CTCF binding sites close to it, which were proposed to carry the imprint for XCI [103,107]. In addition, *DXPas34* or sequences nearby seem important for keeping the Xm active in extraembryonic lineages [99], possibly through the regulation of *Tsix* expression. Other CTCF and YY1 binding sites have been characterized in the XIC, notably in the *Xist* promoter region [106,108,109]. As inter-chromosomal and intra-chromosomal interactions, as well as nuclear localization play a role in random XCI [109–114], it will be interesting to see if it is also the case during imprinted XCI and if it involves CTCF.

Random XCI is found in several eutherian species, including human, but less is known about imprinted XCI. In addition to mouse, imprinted XCI is found in the bovine placenta [115]. In marsupials, imprinted XCI of the Xp was reported both in extraembryonic and somatic tissues [116–118] but the degree of silencing of certain genes vary between individuals and cell types, and during development [119,120]. On the Xp, histones are hypoacetylated but promoters are not methylated [121,122]. Furthermore, *Xist* is poorly conserved and its orthologue, *Lnx3*, encodes a protein, thus it is unlikely to participate in XCI [123]. However, meiotic sex chromosome inactivation during spermatogenesis in marsupials is more stable than in eutherians and could be an alternative way to initiate XCI [124,125]. If so, imprinted XCI may have evolved independently in eutherians and marsupials, or eutherians could have developed new mechanisms to initiate XCI in the early embryo from a template mechanism still used in marsupials [126].

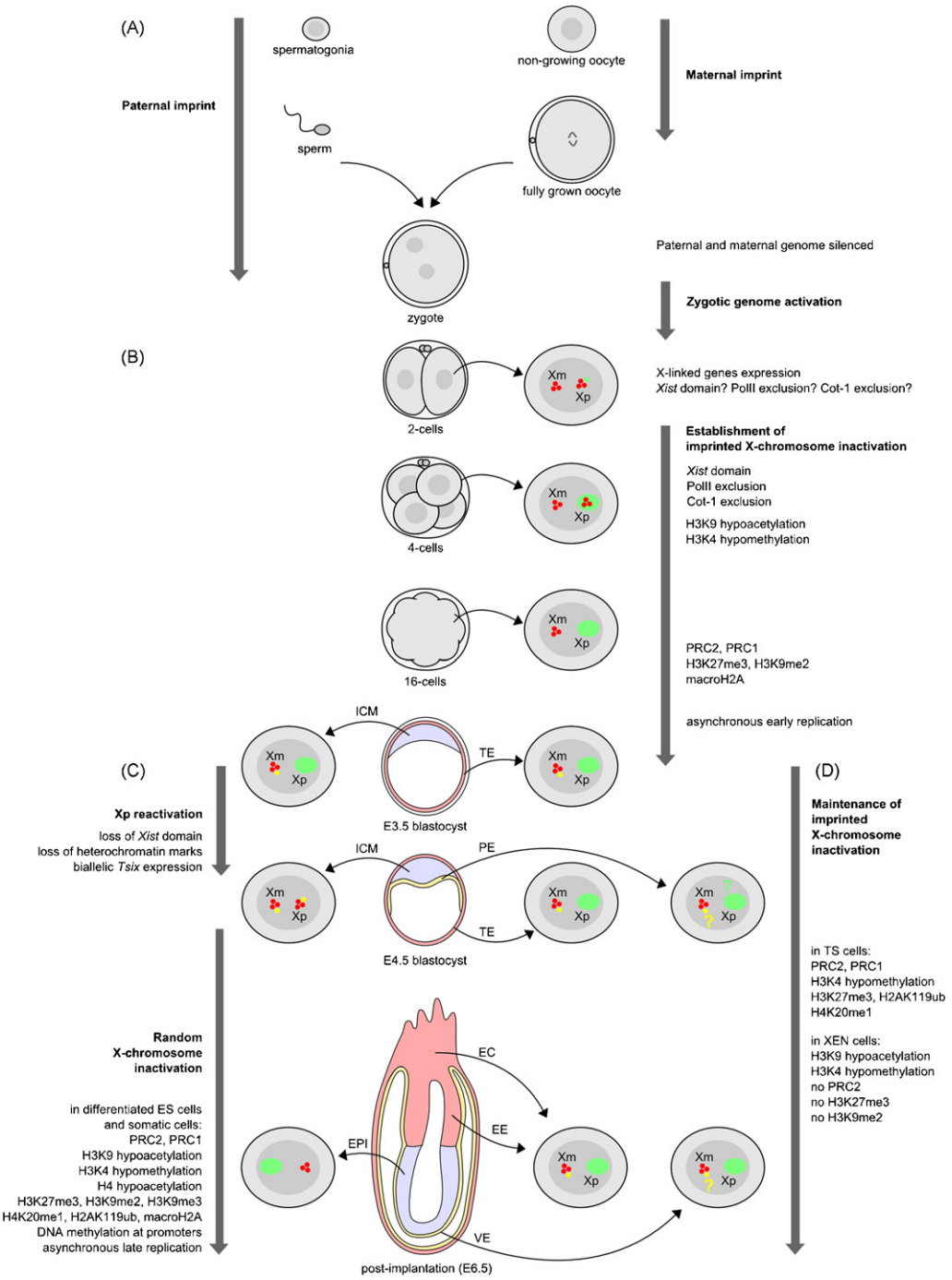

Fig. 3. Kinetics of X-chromosome inactivation during development in mice. Stages after zygotic genome activation are represented together with a schematic view of the XCI pattern. *Xist* domain is depicted by a green oval. *Xist*, *Tsix* and X-linked genes expression are depicted by green, yellow and red dots, respectively. (A) Acquisition of a maternal imprint during oocyte growth and of a paternal imprint either during spermatogenesis or early after fertilization. (B) Establishment of imprinted X-chromosome inactivation (XCI). The marks on the paternal Xp are indicated along the arrow to reflect when they start to be acquired. Cot-1 exclusion refers to the absence of labelling of essentially intergenic transcripts by RNA fluorescence *in situ* hybridization using a Cot-1 probe. Whether the X-chromosome is preinactivated at the two-cell stage is unresolved [75,77]. *Tsix* RNA is present in a fraction of cells in E3.5 blastocysts but it is not known if the expression is equivalent in the trophectoderm and the ICM. (C) Reactivation and random XCI in the embryonic lineage (blue). In the ICM of E4.5 blastocysts and in ES cells, which are derived from the ICM, the *Xist* domain and the heterochromatin marks on the Xp are lost and *Xist* and *Tsix* are expressed biallelically. However, *Xist* expression is very low and hence not represented here. In the epiblast after E5.5 and in differentiated ES cells, either the paternal or the maternal X-chromosome is inactivated due to random XCI. Known heterochromatin marks associated with the inactive X-chromosome are indicated. (D) Maintenance of imprinted XCI in the trophoblast (light red) and primitive endoderm (yellow) lineages. In the trophoblast lineage, the imprinted pattern of XCI is maintained without interruption. *Tsix* expression in the trophoblast lineage at E6.5 is inferred from its expression pattern at earlier and later stages and from its requirement on Xm early after implantation. In the primitive endoderm lineage, XCI is imprinted, but it is not known if the Xp is continuously inactive or if it is transiently reactivated when the primitive endoderm differentiates from the ICM (green question mark). Also, *Tsix* pattern of expression has not been reported in the primitive endoderm lineage (yellow question mark). Known marks on the Xp are indicated for TS cells [81,91,134,135] and XEN cells [91,136], which are cell models of the trophectoderm and the visceral endoderm, respectively. See text for additional references. Xp, paternal X-chromosome; Xm, maternal X-chromosome; ICM, inner cell mass; TE, trophectoderm; EPI, epiblast; EC, ectoplacental cone; EE, extraembryonic ectoderm; ES cells, embryonic stem cells; TS cells, trophoblast stem cells; XEN cells, extraembryonic stem cells; PRC1, Polycomb-Repressive Complex 1; PRC2, Polycomb-Repressive Complex 2.

In human, it is not clear whether the Xp is preferentially inactivated in extraembryonic tissues [127]. In addition, *XIST* seems to be biallelically expressed from the two-cell to the blastocyst stage, arguing against imprinted XCI [128,129]. Contrary to mouse, *TSIX* transcripts in human overlap with only part of the *XIST* gene, which may result in the inability to repress *XIST* in *cis* [130]. Finally, human XmXmXp females and XmXmY males have less severe developmental defects than their mouse counterparts [131], suggesting that a strong maternal imprint does not exist. Taken together, these observations make the existence of imprinted XCI unlikely in humans. Thus, in contrast to autosomal imprinted genes, where imprinting is widely conserved, imprinted XCI appears species-specific.

5. Conclusions and future directions

In the past decade much has been learned with respect to the identity of imprinted genes, imprinting control regions, the epigenetic modifications characteristic of this unusual class of genes and the role that insulators and ncRNAs play in mediating imprinting across large clusters. Nevertheless, our knowledge of how insulators and long ncRNAs work at these loci is rudimentary and a lot remains to be learned. For example, how do ncRNAs silence nonoverlapping genes? What role do cohesins play, if any, in the regulation of insulators? Furthermore, not every large cluster of imprinted genes falls into one of the two distinct categories described in this review. An example of this was described above for the *Kcnq1* locus, where *Cdkn1c* is not solely regulated by expression of the ncRNA Kcnqt1ot1. Here, it is possible that *Cdkn1c* imprinting is also regulated by a CTCF-dependent insulator.

Finally, it is likely that other undiscovered paradigms for imprinting regulation exist. The *Dlk1*/*Gtl2* locus provides one such example. A paternally methylated germline ICR, designated IG-DMR1, is a few kilobase pairs upstream from the start of *Gtl2* transcription at this locus. When this ICR is deleted, the expression on the maternal allele is disrupted but the paternal allele exhibits normal imprinting [7]. It is presently unclear how this allele regulated. An additional example comes from a recent report by Wood and colleagues where allele-specific differences in a co-transcriptional process were described [132]. In this study the newly identified *H13* imprinted gene was shown to use alternate polyadenylation (polyA) signals to govern allele-specific expression of alternate transcripts. The choice of polyA sites was dictated by allele-specific methylation of a CpG island located between the sites, with DNA methylation of the island repressing the use of the adjacent set of polyA signals and the readthrough to the downstream polyA signals. Thus, it seems reasonable to predict that other such intriguing regulatory processes will emerge in the study of imprinted genes.

Conflict of interest

The authors declare that there are no conflicts of interest.

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