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Epigenetic regulation of mammalian genomic imprinting

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Imprinted genes play important roles in development, and most are clustered in large domains. Their allelic repression is regulated by ‘imprinting control regions’ (ICRs), which are methylated on one of the two parental alleles. Non-histone proteins and nearby sequence elements influence the establishment of this differential methylation during gametogenesis. DNA methylation, histone modifications, and also polycomb group proteins are important for the somatic maintenance of imprinting. The way ICRs regulate imprinting differs between domains. At some, the ICR constitutes an insulator that prevents promoter–enhancer interactions, when unmethylated. At other domains, non-coding RNAs could be involved, possibly by attracting chromatin-modifying complexes. The latter silencing mechanism has similarities with X-chromosome inactivation.

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Abbreviations

Ascl2	Achaete–scute-like 2
AS	Angelman syndrome
CTCF	CCCTC-binding factor
DNMT	DNA methyltransferase
Eed	embryonic ectoderm development
HDAC	histone deacetylase
ICR	imprinting control region
Igf2	insulin-like growth factor-2
Ipl	imprinted in placenta and liver
MBD	methyl-CpG-binding domain
PcG	Polycomb group
PGC	primordial germ cell
PRC	Polycomb repressive complex
PWS	Prader–Willi syndrome
RNAi	RNA-interference

Introduction

In placental mammals, certain genes are subject to imprinting, such that their expression depends on whether they are inherited from either the mother or the father. Genomic imprinting was discovered twenty years ago, as an outcome of nuclear transplantation experiments in the mouse [1,2]. About eighty genes are now known to be

imprinted in mice and humans, and imprinting is conserved in ruminant species as well [3–6]. Many imprinted genes are involved in foetal development and growth, and some influence behaviour [4–7]. Imprinting appears to be particularly important for placental development [8*,9,10*].

Most imprinted genes are organised in large chromosomal domains [3]. Transgenic studies have identified sequence elements in these domains that are essential for the imprinted gene expression. These ‘imprinting control regions’ (ICRs) are regulated by epigenetic modifications (Figure 1). ICRs are up to several kilobases in length, and are rich in CpG dinucleotides (many correspond to CpG-islands). Another hallmark of ICRs is that they have DNA methylation on one of the two parental alleles. At most ICRs, the allelic methylation originates from the egg. At only a few, it is established during spermatogenesis. Following fertilisation, allelic methylation marks are maintained throughout development and they mediate imprinted expression [4,7].

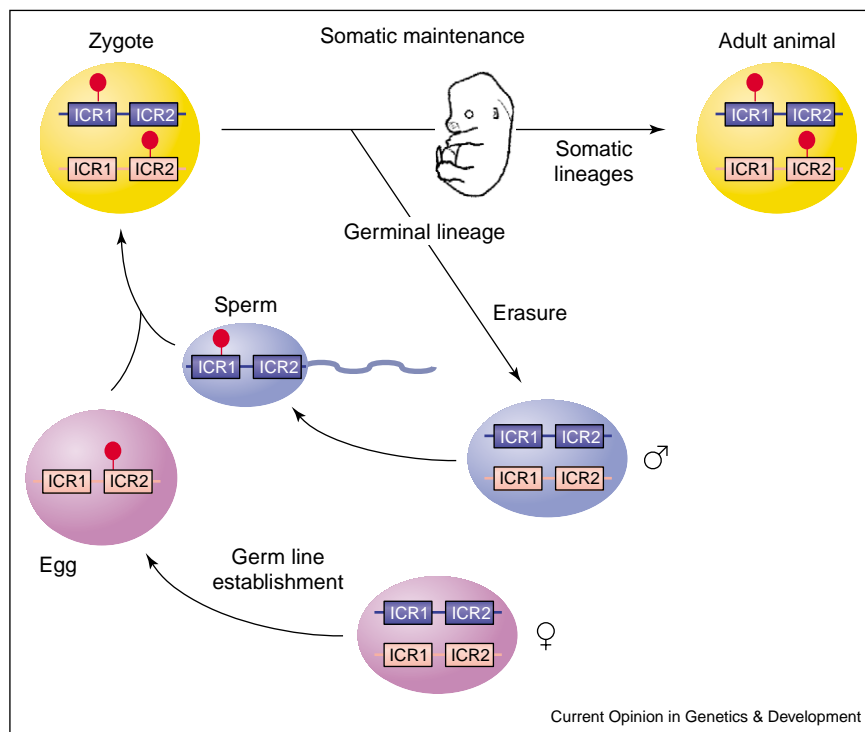
In this review, we focus on novel insights into the germ line establishment and the somatic maintenance of epigenetic marks at ICRs. For most imprinted domains, it remains poorly understood how the ICR brings about allelic gene repression. Nevertheless, recent studies suggest that at some imprinted domains non-coding RNAs could be involved in this developmentally-regulated ‘reading’ of imprints.

Germ-line establishment of imprints

Before the acquisition of methylation at ICRs during spermatogenesis or oogenesis, pre-existing methylation needs to be erased in the primordial germ cells (PGCs). This appears to be an active demethylation process, involving yet-unknown enzymatic activities [11].

Novel imprints are established much later during gametogenesis, at late foetal stages in male germ cells, or after birth in growing oocytes. A protein sharing homology with DNA methyltransferases (DNMTs), Dnmt3l, is essential for the acquisition of methylation in oocytes [12,13*]. Dnmt3l lacks a catalytic methyltransferase domain, but could direct methylation to ICRs via interaction with the *de novo* methyltransferase Dnmt3a [13*,14]. Using a conditional targeting approach, it has been confirmed that Dnmt3a (but not Dnmt3b) is the methyltransferase that puts methyl groups onto ICRs, for some in the female and for others in the male germ line (H Sasaki, personal communication). However, because *Dnmt3a* and *Dnmt3l* are expressed in both the germ lines, they cannot explain

Figure 1



Parental imprints are established during oogenesis, or spermatogenesis, at sequence elements that control the imprinted expression (the 'imprinting control regions' [ICRs]). After fertilisation of the egg by the sperm, these imprints are maintained throughout development. DNA methylation (lollypops) is the most consistent hallmark of imprints. Two examples of ICRs are depicted: an ICR with paternally-derived (ICR1), and one with maternally-derived DNA methylation (ICR2).

why ICRs acquire DNA methylation in either the egg or the sperm.

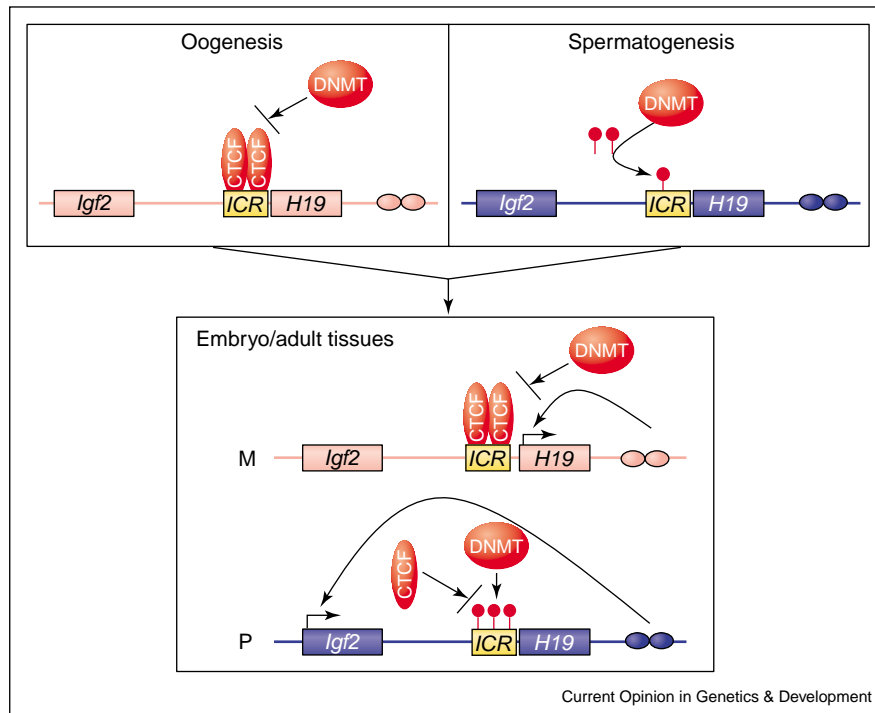
One possibility is that the binding of non-histone proteins to individual ICRs could prevent acquisition of methylation in one of the two germ lines. This seems the likely scenario at the ICR that controls the *Insulin-like growth factor-2* (*Igf2*) and *H19* genes on distal mouse chromosome 7 (Figure 2). The zinc-finger protein CTCF (CCCTC-binding factor) binds to its unmethylated maternal allele in somatic cells. In two recent studies, point mutations were introduced into the ICR to prevent binding of CTCF [15,16]. Upon passage through the female germ line, this led to acquisition of aberrant methylation on the maternal allele. The hypothesis that CTCF could prevent DNA methylation in the female germ line was confirmed by a recent study in which CTCF expression was downregulated during oogenesis using a transgenic RNA-interference (RNAi) approach [17]. It is unclear, at present, whether in the male germ line there could be factors directing the DNA methylation to this ICR [18], or whether the methylated state is established by default.

Different proteins could be involved in the sex-specific acquisition of methylation at other ICRs. The imprinted

IGF2-receptor (*Igf2r*) gene in the mouse has an intronic ICR that is methylated on the maternally inherited allele [19,20]. Transgenic experiments pinpointed a short sequence motif that could protect the paternal copy of the ICR from becoming methylated. Moreover, binding of a nuclear protein to this motif was demonstrated [21].

Nearby sequences can influence the establishment of methylation at ICRs as well. The *Rasgrf1* locus on mouse chromosome 9 is methylated on the expressed paternal allele, at a region located 30 kb upstream of the promoter (Figure 3a). A direct repeat sequence, adjacent to this putative ICR, is essential for the establishment of methylation during spermatogenesis and is not present in species in which the gene is not imprinted [22]. It was reported that, when the ICR of *Igf2r* replaces the endogenous *Rasgrf1* repeats, it can induce paternal methylation at the ICR of the *Rasgrf1* gene [23]. Hence, an unrelated nearby sequence is able to induce paternal methylation at the *Rasgrf1* imprinting control region. A mechanism comparable to that of *Rasgrf1* could, maybe, explain the species-specific patterns of imprinting at the growth-factor receptor binding protein-10 gene, a regulator of foetal growth [24,25].

Figure 2



The establishment and maintenance of DNA methylation at the ICR of the *Igf2-H19* locus. This ICR acquires methylation (lollipops) during late spermatogenesis. In the female germ line, by contrast, the ICR is protected against methylation by the zinc-finger protein CTCF. After fertilisation, and throughout development, the maternal allele (M, pink) continues to be protected against methylation by CTCF binding. CTCF binding onto the maternal chromosome creates a chromatin boundary that prevents interaction between the *Igf2* gene and enhancers (ovals) located downstream of *H19*. The methylation on the paternal allele (P, blue) is maintained throughout development and prevents CTCF binding. On the paternal chromosome, the *Igf2* promoters can therefore interact with the enhancers, but here the *H19* gene is repressed because of the ICR methylation spreading into the nearby *H19* promoter.

ICR methylation at a domain on human chromosome 15 is influenced by nearby sequences as well. This large imprinted domain is deregulated in two neuro-developmental diseases, Prader-Willi syndrome (PWS) and Angelman syndrome (AS) [4]. Its ICR is located at the 5'-portion of the *SNRPN* gene (Figure 3b). Genetic and epigenetic alterations at this ICR give rise to the PWS syndrome [26]. Both in humans and mice, the ICR at *SNRPN* has maternal DNA methylation, established upon transmission through the female germ line [27*,28]. At 35 kb upstream of the ICR there is a region which, when deleted, gives rise to AS syndrome. Recently, it was demonstrated that this far-upstream region is essential for the establishment of the maternal DNA methylation at the *SNRPN* ICR [27*].

It remains to be explored how specific repeat sequences can induce DNA methylation at nearby ICRs, and also whether other flanking-sequences influence this process. One possibility could be that certain (repeat) sequences recruit *trans*-acting factors in one of the germ lines only, and thereby, locally induce methylation.

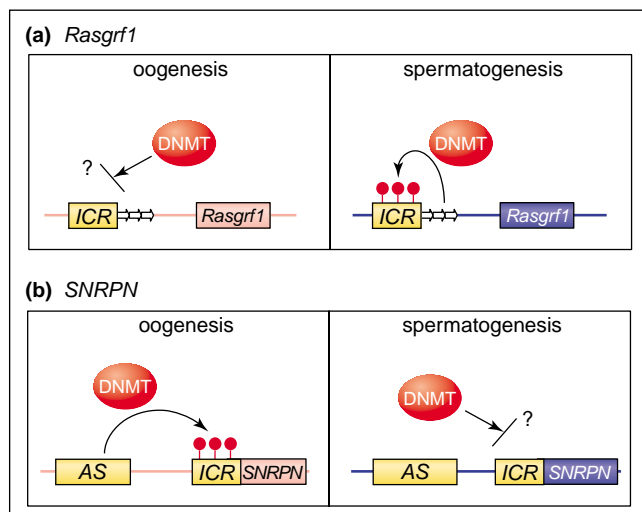
Another interesting possibility to be explored is whether the establishment of methylation involves micro-RNAs via an RNAi mechanism, as recently observed for repeat sequences at pericentric heterochromatin [29]. Intriguingly, micro-RNAs were discovered recently at the imprinted *Dlk1-Gtl2* cluster on mouse chromosome 12, but their precise role is yet to be unravelled [10*,30*].

Somatic maintenance during development

Epigenetic deregulation of imprinting may lead to complex diseases in humans and is also observed frequently in cancer [26,31,32]. Embryo culture, somatic cell nuclear transfer and *in vitro* fertilisation procedures can also affect imprinting [6,33-35]. Other environmental factors, such as the folate content of the diet [36*], may disrupt imprinting as well. Despite the many examples of its epigenetic deregulation, our understanding of how imprinting is normally maintained in somatic cells remains limited.

Continuous expression of the maintenance methyltransferase Dnmt1 is clearly essential for the maintenance of

Figure 3



Sequence elements are important for the sex-specific establishment of DNA methylation at ICRs. **(a)** At the ICR controlling the mouse *Rasgrf1* locus, the male-specific establishment of methylation requires the presence of neighbouring repeat sequences (open arrows).

(b) The ICR at the *SNRPN* gene regulates imprinting along a large chromosomal domain, which is deregulated in the Prader-Willi syndrome (PWS). For the acquisition of methylation at the ICR in the female germ line, a region located >30 kb further upstream (the AS region) is essential.

imprinting [37]. Levels of *Dnmt1* are also critical, and overexpression in embryos was found to give rise to methylation on normally unmethylated alleles of ICRs [38]. However, during pre-implantation the parental genomes are subject to widespread demethylation and, at later stages, they acquire high levels of *de novo* DNA methylation [4,37]. It is unknown why ICRs are fully resistant to these global methylation changes. One possibility could be that the maintenance of ICR methylation is linked to the difference in replication timing between the parental chromosomes at imprinted domains [39]. Yet, at the imprinted *Igf2-H19* domain, asynchronous replication timing was found recently not to be linked to the differential methylation at the ICR [40,41].

Also, non-histone proteins could be important for the somatic maintenance of imprinting. At the ICR regulating the *Igf2-H19* imprinted locus, for instance, CTCF protects the unmethylated maternal allele against the acquisition of DNA methylation [15,16,17^{*}]. Furthermore, at *Peg3* (another imprinted locus, on mouse chromosome 7), methylation-sensitive binding of a DNA-binding protein has been observed [42].

Conversely, on the methylated allele of ICRs, the association of methyl-CpG-binding domain (MBD) and other proteins could play a role in keeping the DNA methylated [18,43^{*},44]. MBD proteins can locally

recruit chromatin-remodelling activities and histone deacetylases (HDACs) [37] and were found to be associated with the methylated allele of the imprinted *U2af1-rs1* gene [43^{*},45]. To date, however, there is no genetic evidence that MBD proteins regulate imprinting [46].

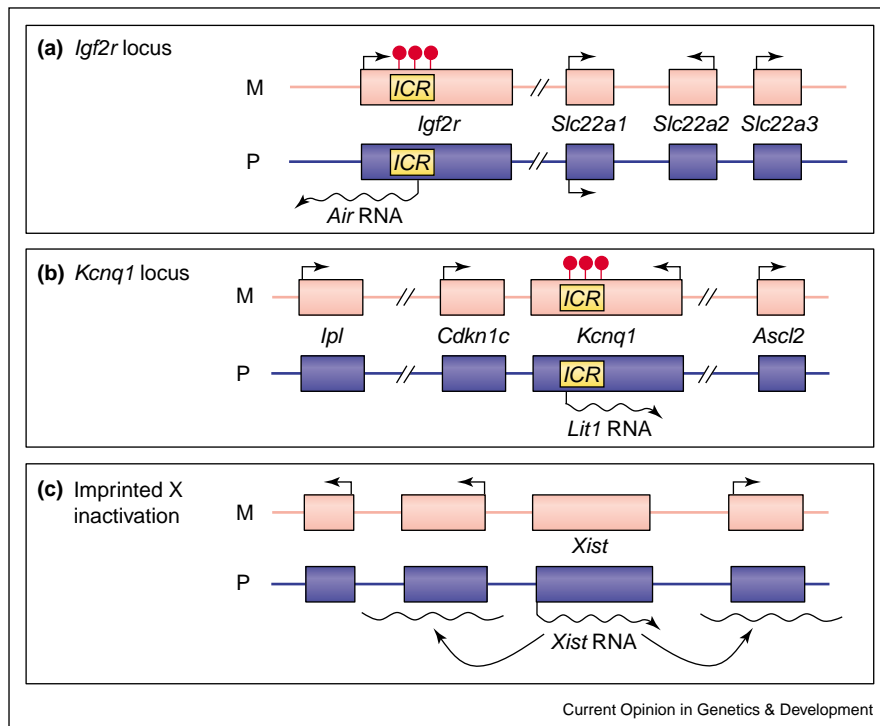
Another possible mechanism related to the regulation of ICRs is the presence of different histone modifications on the chromatin associated with the methylated allele and the chromatin associated with the unmethylated allele. On the allele that carries DNA methylation, the ICRs analysed display H3-lysine 9 (K9) methylation and hypoacetylation on histones H3 and H4. On the allele without DNA methylation, by contrast, chromatin is hyper-acetylated and has H3-K4 methylation [43^{*},44,45]. These covalent modifications were detected in all cells and tissues analysed and could be linked to the maintenance of the differential DNA methylation [32,37,43^{*},44]. Underlying enzymatic activities are still to be discovered, but could in part be specific to imprinted domains. Compared to bulk chromatin, for instance, ICRs are highly resistant to treatment with TSA, an inhibitor of HDACs. This could indicate that histone acetylation is regulated differently at ICRs, compared to the genome on the whole [47].

Allele-specific gene repression along imprinted domains

The way ICRs give rise to imprinted expression differs between domains. One of the best-studied examples is the *Igf2-H19* domain (Figure 2). Its ICR is bound by CTCF and functions as a chromatin insulator on the unmethylated maternal allele, preventing interaction between promoters on one side, and enhancers on the other [15,16,17^{*}]. Several other imprinted domains are suspected to be regulated by methylation-sensitive chromatin insulators as well [25,42].

The intronic ICR of the mouse *Igf2r* gene is methylated on the maternal allele. It was shown that this ICR is essential for the repression of *Igf2r* on the paternal chromosome. In the extra-embryonic tissues it represses also the paternal alleles of two other genes, *Slc22a2* and *Slc22a3*, located several hundreds of kilobases away [19,20^{**}]. In contrast to the paternal repression of the *Igf2r* promoter, the paternal repression of *Slc22a2* and *Slc22a3* in the placenta does not involve DNA methylation [19]. Interestingly, the ICR produces a non-coding RNA, called *Air*, from its unmethylated paternal allele. To test whether this non-coding RNA could be involved in the paternal repression along the domain, a premature poly-adenylation signal was introduced into the ICR, giving rise to a short, truncated *Air* transcript. This abolished not only the paternal repression of *Igf2r*, but also that of *Slc22a2* and *Slc22a3*, suggesting that *Air* mediates repression along the entire domain [20^{**}].

Figure 4



At the ICRs controlling (a) the *Igf2r* and (b) the *Kcnq1* domains, non-coding RNAs are produced from the unmethylated paternal allele. These non-coding RNAs (*Air* at *Igf2r*, and *Lit1* at *Kcnq1*) could be involved in the paternal repression along these domains, which extends furthest in the placenta at both the domains. This developmentally-regulated silencing mechanism bears similarities with (c) imprinted X-chromosome inactivation in the mouse placenta, which is brought about by 'coating' of the paternal X-chromosome by the non-coding *Xist* transcript, and subsequent recruitment of chromatin-modifying complexes.

A similar silencing mechanism, involving non-coding RNA, could be responsible for paternal repression along the *Kcnq1* (potassium voltage-gated channel, subfamily Q, member 1) domain on distal mouse chromosome 7 (Figure 4b). Several genes in the domain, including *Ascl2* (*Achaete-scute-like 2*) and *Ipl* (*imprinted in placenta and liver*), are imprinted in the placenta and are important for its development [9,48]. The ICR controlling this domain is maternally methylated and produces a non-coding RNA, *Lit1*, from its unmethylated paternal allele [49]. Like the *Igf2r* ICR, the ICR of the *Kcnq1* domain functions as a silencer and is essential for gene silencing along the paternal chromosome [50,51]. It is not yet known whether *Lit1* RNA mediates this repression. The paternal repression at *Ipl* and *Ascl2*, and at other genes in the cluster, does not seem to imply DNA methylation [48,49]. As at the *Igf2r* locus, a chromatin-mediated silencing mechanism is therefore likely to be involved.

It was shown recently that the Polycomb group (PcG) protein Eed (embryonic ectoderm development) is essential for allelic gene repression at the *Kcnq1* and several other imprinted domains [52]. PcG proteins are organised in 'Polycomb repressive complexes' (PRCs) that

play important roles in maintaining genes in a repressed state [53]. Eed is part of PRC2, an early-embryonic PRC that also comprises Ezh2 (Enhancer of zeste-2, also called Enx1 in the mouse), a histone methyltransferase that adds methyl groups to K9 and K27 on histone H3. The PRC2 complex can also interact with HDACs [53]. Research from our laboratory indicates that in early extra-embryonic development, there is H3-K27 and H3-K9 methylation along the repressed paternal *Kcnq1* domain (D Umlauf, personal communication). Whether this differential histone H3 methylation is regulated by the PRC2 complex and whether *Lit1* RNA plays a role in it, is yet to be investigated.

The allelic repression along the imprinted *Igf2r* and the *Kcnq1* domains has marked similarities with X-chromosome inactivation [37]. For instance, Eed is required not only for maintaining repression at several autosomal imprinted genes [52], but is also essential for imprinted X-chromosome inactivation [54]. In the extra-embryonic lineages, the paternal X is inactivated, whereas the maternal X is active. In *Eed*^{-/-} mouse conceptuses, the paternal X chromosome was found not to be repressed. X-inactivation is controlled by the *Xist* gene (X-inactive specific transcript), which produces a non-coding RNA that paints

the chromosome *in cis* and brings about the X-inactivation (Figure 4c). It was reported in several recent studies that Eed and Ezh2 (Enx1) are associated with the inactive X chromosome. This induces H3-K27 and H3-K9 methylation and is thought to be involved in establishing X-inactivation [55,56]. These novel findings raise the issue of whether PcG proteins could be transiently associated with the *Igf2r*, *Kcnq1* and other imprinted domains as well, and be involved in their lineage-specific patterns of imprinting.

Conclusions

Last year's research has greatly enhanced our understanding of imprinting. Mouse studies further emphasised the diverse developmental roles of imprinting, and some imprinted genes were found to be particularly important for placental development. As for the establishment of methylation at ICRs, we now know which DNMTs are involved. CTCF and other nuclear proteins could prevent the establishment of DNA methylation in one of the two germ lines. Yet, how DNMTs are targeted to the ICR in the opposite germ line, and via which mechanisms nearby sequences can mediate this process, remains unknown. One hypothesis would be that specific sequence elements in the vicinity of ICRs are transcribed in one of the two germ lines only. Possibly via an RNAi-mediated mechanism, germ-line-specific RNAs produced in this manner could locally attract DNA methylation and compacted chromatin.

Together with the allelic DNA methylation, non-histone proteins and covalent histone modifications are likely to be involved in the somatic maintenance of imprinting. For some imprinted domains, PcG proteins are also essential. An important outstanding issue is how these different epigenetic features are linked during development, and by which enzymatic complexes they are regulated.

The recent studies also unravelled similarities between the paternal repression at the *Igf2r* locus and that along the *Kcnq1* domain. It should be relevant to explore in more detail to what extent these silencing mechanisms are comparable to X chromosome inactivation. One of the emerging questions is whether, like in X-inactivation, non-coding RNAs are involved in the repression *in cis* along these and other imprinted domains. If so, do these non-coding RNAs function by attracting PRC complexes (and other chromatin-modifying activities) and thereby give rise to gene repression? Furthermore, could the chromatin regulatory complexes involved be developmentally regulated? This could explain, for instance, why the allelic repression along the *Kcnq1* and *Igf2r* domains extends furthest in the placenta. With so many unresolved issues, some of which were touched upon only briefly in this review (but that are discussed in more detail elsewhere [10[•],23,30[•],36[•]]), the coming year of imprinting research promises to be an exciting one.

Acknowledgements

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The mouse *Igf2r* imprinting cluster comprises several genes — *Igf2r*, *Slc22a2*, and *Slc22a3* — that are repressed on the paternal chromosome. The intronic ICR in the *Igf2r* gene is essential for this paternal repression [19]. The ICR comprises the promoter for a paternally-expressed non-coding RNA, called *Air*. By introducing a premature poly-adenylation signal, leading to a truncated *Air* transcript, the authors show that this non-coding RNA is important for the silencing of the *Igf2r*, *Slc22a2*, and *Slc22a3* genes on the paternal chromosome.

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It was found in this study for the ICR of the PWS domain on human chromosome 15q11-q13 that nearby sequences are essential for the establishment of its differential DNA methylation. Particularly, a region located 35kb upstream of the ICR — the minimal AS deletion region — was shown to be essential for the acquisition of the maternal methylation at the ICR in the female germ line. Together with [22], this work demonstrates that nearby sequence can influence the germ-line establishment of DNA methylation at ICRs.

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