Chromosome Loops, Insulators, and Histone Methylation: New Insights into Regulation of Imprinting in Clusters

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Imprinted genes in mammals are organized into clusters in which genes share regulatory elements. *Igf2* and *H19* are separated by 100 kb (kilobases) of DNA, and both genes use enhancers that are located distal to *H19*. Alternate access to the enhancers by the two genes is in part regulated by a CTCF-dependent insulator located upstream of *H19*. We find that differentially methylated regions in both genes interact physically over the 100-kb distance. These interactions are epigenetically regulated and partition maternal and paternal chromatin into distinct loops. This creates a simple epigenetic switch for *Igf2* whereby it moves between an active and a silent chromatin domain. In the adjacent *Komm3cl* cluster, by contrast, a noncoding RNA gene is flanked by several silent genes, which are marked by repressive histone modifications. Histone methylation is targeted directly or indirectly to the region by the noncoding RNA and is maintained in the absence of DNA methylation. We propose that imprinting regulation in clusters falls into different categories. The “insulator-loop” model may also be applicable to *Dlk1/Gtl2* and *Rasgrf1*.

Genomic imprinting was discovered 20 years ago (McGrath and Solter 1984; Surani et al. 1984). The realization of developmental nonequivalence of the two parental genomes (the maternal and the paternal one) was quickly followed by the insight that epigenetic mechanisms such as DNA methylation are key to this parental chromosomal identity (Sasaki et al. 1992; Brandes et al. 1993; Stoger et al. 1993; Ferguson-Smith and Surani 2001; Reik and Walter 2001; Sleutels and Barlow 2002; Verona et al. 2003; Delaval and Feil 2004). At the same time, the “genetic conflict” hypothesis of evolution of imprinting was conceived (Haig and Westoby 1989), followed by the identification of the first imprinted genes in mice and humans (Barlow et al. 1991; Bartolomei et al. 1991; DeChiara et al. 1991). By the mid-1990s, therefore, some of the cornerstones of imprinting research were in place. Recent significant additions to this puzzle are experiments showing how imprints are introduced in the parental germ cells by DNA methyltransferases and their cofactors (Bourc’his et al. 2001; Hata et al. 2002; Kaneda et al. 2004). How imprints are erased in early germ cells is still a mystery that needs to be explored.

Meanwhile, a large amount of information has been accumulated on individual imprinted genes (currently about 80 in mouse) and their functions in fetal growth control, neonatal physiology, and adult behavior (http://www.mgu.har.mrc.ac.uk/research/imprinting/). Once a number of imprinted genes had been isolated, it quickly became clear that most imprinted genes are clustered in the genome. Some of the larger clusters extend over 1–2 Mb (megabases) and contain several imprinted genes. The reason why imprinted genes are clustered in this way and the evolution of clusters is still not understood. However, it can be imagined that genetic conflict occurs at the level of gene products as well as at different mechanistic levels. For example, maternal expression of the *H19* gene prevents paternal expression of the linked growth-suppressing *Igf2* gene. These mechanistic interactions ("battles") between imprinted genes are facilitated by physical linkage of the genes and shared regulatory elements.

Clustering and sharing of regulatory elements is indeed a common theme in imprinting. Such elements include enhancers, silencers, insulators, and activators, all of which have the potential to be epigenetically regulated. Of particular interest are the so-called “imprinting centers” (ICs), which have the property that they regulate epigenetic modifications and imprinted expression of several genes throughout clusters. Their influence can extend over several hundred kilobases to megabases.

The purpose of this paper is to explore the nature of mechanistic interactions between imprinted genes in clusters. We describe recent insights into the *Igf2-H19* region suggesting that reciprocal access to a single set of enhancers is regulated by chromatin “looping.”
jacent imprinted domain, we argue that an imprinted non-coding transcript, \textit{Kcnq1ot1}, is pivotal for the recruitment of repressive histone modifications leading to a silent chromatin structure in the adjacent genes. We further explore the general applicability of these two models of imprinting regulation to other imprinting clusters.

**THE IGF2–H19 LOCUS (IC1 DOMAIN)**

Structure of the Locus and Regulatory Elements

The fetal growth factor Insulin-like growth factor 2 gene (\textit{Igf2}) is paternally expressed and lies upstream of the maternally expressed noncoding RNA gene \textit{H19} (Fig. 1). The distance between \textit{Igf2} and \textit{H19} is \(~100\) kb. While the two genes are expressed from the opposite parental chromosomes, their spatial and temporal patterns of expression are strikingly similar. Both genes are expressed predominantly in mesodermal, endodermal, and extraembryonic tissues in the developing fetus, with a decline of expression during the first 3 weeks of postnatal life.

Lineage- and tissue-specific expression of both genes are governed by a number of different enhancers, most of which are located distal to \textit{H19} (Leighton et al. 1995; Kaffer et al. 2001; Davies et al. 2002). A differentially methylated region (DMR) with paternally derived germ line methylation is located 2 kb upstream of \textit{H19}. This region acts as an insulator or boundary element when unmethylated; it has multiple binding sites for the insulator protein CTCF, which is bound to the maternal allele (Bell and Felsenfeld 2000; Hark et al. 2000; Kanduri et al. 2000; Szabo et al. 2000; Arney 2003). CTCF binding is sensitive to DNA methylation, so the methylated paternal allele of the DMR does not bind CTCF. As a result, the insulator is inactive. The active insulator on the paternal allele is thought to restrict access of the enhancers exclusively to the \textit{H19} promoter. On the paternal allele, by contrast, the \textit{H19} promoter is methylated and so this gene is silent, but the insulator is now inactive, allowing the distal enhancers access to the \textit{Igf2} gene. Notably, the \textit{Igf2} promoters are not generally methylated on the inactive allele, and even show DNase hypersensitive sites, indicating that in principle the chromatin is transcriptionally permissive (Feil et al. 1995).

In addition to the distal enhancers and the insulator region, regulatory elements in \textit{Igf2} are also important (Arney 2003). The \textit{Igf2} gene has three DMRs: DMR0 is maternally methylated and overlaps with the placental specific \textit{Igf2} promoter P0; DMR1 is paternally methylated and contains a methylation-sensitive silencer (Constancia et al. 2000; Eden et al. 2001); and the intragenic DMR2 is also paternally methylated and contains a methylation-sensitive activator (Murrell et al. 2001). These three DMRs are non-germ-line DMRs in that their differential methylation arises during early postimplantation development (Lopes et al. 2003). Importantly, deletion of the unmethylated DMR1 does lead to reactivation of the otherwise silent maternal \textit{Igf2} allele, in the presence of an apparently intact insulator (Constancia et al. 2000). Additional silencer and enhancer sequences are present in the locus; a more complete summary can be found in Arney (2003).

**Models for Igf2–H19 Imprinting**

An initial model for imprinting regulation in the locus was based on enhancer competition, whereby \textit{H19} had privileged access to the enhancers, but when \textit{H19} was methylated, \textit{Igf2} could gain access to them (Bartolomei 1995a; Thompson et al. 1995). However, this model does not account for the differential methylation of different DMRs, which arise during early postimplantation development. An alternative model, based on the role of the \textit{Kcnq1ot1} transcript, suggests that this transcript acts as a repressive element, leading to a silent chromatin structure in the adjacent genes.
and Tilghman 1992]). This model was superseded by the current one in which the H19 DMR is shown to have methylation-sensitive insulator function, which depends on CTCF binding. How do insulators work? They are defined as elements that block promoter enhancer communication (Labrador and Corces 2002); recently CTCF has been found to bind nucleoplasmin, which itself appears to be tethered to the nuclear surface (Yasufray et al. 2004). Such an anchoring of insulators to nuclear substructures could lead to the formation of chromatin loops that separate promoters and enhancers so that they can no longer interact (Fig. 2). Interestingly, a chromatin loop model was proposed some time ago for Igf2 and H19, in which they come into close physical proximity, with different contact points on the maternal and paternal chromosomes (Fig. 2) (Banerjee and Smallwood 1995).

Another issue that needs to be considered in this context is the observation that epigenetic marks in clusters can be under the control of imprinting centers. Thus in the PWS IC on the paternal allele the methylated H19 DMR (silenced) is bound by CTCF and possibly other proteins (stippled ovals), and Igf2 DMR1 interact, resulting in two chromatin domains, with the H19 gene in an active domain with its enhancers (small circles) close to its promoter, and the Igf2 gene in an inactive domain away from the enhancers (shaded area). On the paternal allele the methylated H19 DMR associates with the methylated Igf2 DMR2 through putative protein factors (filled ovals), moving Igf2 into the active chromatin domain. The location of DMR2 at the end of the Igf2 gene positions its promoters in close vicinity to the enhancers downstream of H19. H19 remains in the active domain but is silenced by DNA methylation.

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Testing the Loop Model

Chromosome looping was proposed many years ago to underlie the action of distant enhancer elements on proximal promoters, especially in more complex vertebrate genomes (Bulger and Groudine 1999). Only recently, however, have technological developments allowed these models to be directly tested. The first method, chromosome conformation capture (3C), is based on cutting cross-linked chromatin with defined restriction enzymes, and religating the ends under very dilute DNA concentration (Dekker et al. 2002). Under these conditions, restriction sites from remote genomic regions can ligate to each other if they were in close physical proximity when the chromatin was cross-linked. Thus, successful PCR amplification with primers from the two remote regions would indicate physical proximity of the remote regions. In the mammalian system, this technique has been applied to study the developmental regulation of beta globin gene expression (Tolhuis et al. 2002). A remote enhancer (the LCR) has indeed been shown to come into close physical contact with the gene with the intervening DNA looping out. These interactions are dynamic and are regulated in a tissue and developmental fashion (Palstra et al. 2003).

The second method has also been developed to look at beta globin expression and is based on cross-linking locally an RNA FISH probe to the nuclear RNA as it is transcribed (of the globin gene in fetal blood cells in this case), followed by pulldown of the crosslinked protein-nucleic acid complex using a tag on the FISH probe. The complex is then analyzed to see which remote sequences are trapped. As with the 3C technique, areas of the enhancer (LCR) were found to contact the gene in tissues in which transcription occurs (Carter et al. 2002). The confirmation of “looping” with different techniques is important, and inspires confidence in these new technologies. We chose to test the looping model for Igf2-H19 by two different techniques. First, we reasoned that chromatin immunoprecipitation (ChIP) could be used with an antibody specific to a protein located on the H19 DMR in
order to see whether areas in Igf2 would be coprecipi-
tated. However, a protein such as CTCF (which is bound
to the maternal H19 DMR) is so ubiquitous that it cannot
be used for this purpose. We thus decided to engineer a
H19 DMR with a unique protein tag. Three binding se-
quences for the yeast Gal4 transcriptional activator pro-
tein (termed UAS) were inserted into the H19 DMR by
homologous recombination in ES cells and knockin mice
were generated (Murrell et al. 2004). These mice were
then bred with transgenic mice ubiquitously expressing
the DNA-binding domain of the Gal4 protein fused to a
unique peptide tag (human MYC). Following ChIP with
a MYC tag antibody, the DMRs and other regions were
analyzed by Q-PCR. The H19 DMR showed strong en-
richment with the antibody, confirming that the knockin
strategy was working and that the Gal4-MYC protein was
indeed located at the H19 DMR with the inserted UAS se-
quences. Interestingly, with maternal transmission of
the modified DMR, DMR1 of Igf2 was also enriched, but nei-
thor DMR2 nor intervening sequences between Igf2 and
H19 were. With paternal transmission, by contrast, nei-
ther DMR1 nor intervening sequences were enriched, but
DMR2 was. The preliminary conclusions from this ex-
perimental system were that on the maternal chromo-
some, the H19 DMR was closely associated with Igf2
DMR1, whereas on the paternal chromosome it was as-
soiated with DMR2.

We applied the 3C method in order to confirm these re-
sults. Ligation products were indeed detected between the
H19 DMR and DMR2, and the H19 DMR and DMR1. For
DMR2, it was possible to determine by sequence polyn-
morphism that it was the paternal allele that was as-
soiated with the H19 DMR. Thus the combined evi-
dence from the different techniques suggests that the H19
DMR comes in close proximity to DMR1 on the maternal
allele and to DMR2 on the paternal one. It should be
noted that these analyses are not quantitative, and so the
proportion of cells in any one tissue that contain the loop
structures remains to be determined. The loops we see
may be only transient and may or may not be present in
normal or tumors cells. It will also be of interest to see
whether other sequences between Igf2 and H19, or those
further away from the genes, particularly Ins2, which is
also paternally expressed in some tissues, come into
physical proximity with the DMRs.

An Epigentic Switch

The model arising from these observations is simple (Fig. 2). On the maternal allele the unmethylated H19
DMR and Igf2 DMR1 come together; proteins involved in
these interactions may well include CTCF and others.
This places Igf2 inside a loop and insulates it from the en-
hancers. On the paternal allele, Igf2 moves out of the loop
since the H19 DMR and DMR2 are now interacting; this
allows interaction between the enhancers and Igf2. While
H19 remains outside of the “silent” loop, it is inactivated
by DNA methylation. CTCF cannot be involved in the in-
teractions between the H19 DMR and DMR2 because it
does not bind to the methylated alleles. Whether proteins
that bind preferentially to methylated DNA such as MBDs
are involved in these interactions needs to be established.
In human fibroblasts the 3C method revealed further
allele-specific interactions between restriction fragments
containing the promoters of Igf2 and H19 and a fragment
carrying their (presumptive) shared enhancers (Fig. 2)
(Y. Yang and M. Higgins, unpubl.). Presumably these en-
hance/promoter interactions are mediated by additional
higher-order chromatin structures, perhaps involving a
putative CTCF-dependent insulator (conserved between
mouse and humans) that lies downstream of the enhancers
(Ishihara and Sasaki 2002). Potential involvement of
CTCF in looping can now be tested genetically using mu-
tant DMRs that lack CTCF-binding sites (Pant et al. 2003;
Schoenherr et al. 2003; Szabo et al. 2004), and whether
methylation of the paternal DMR is needed for looping
can be tested using a mutant DMR that lacks CpGs (M.
Bartolomei, pers. comm.). These experiments may begin
to reveal the cis- and trans-acting requirements for loop-
ing (Patris et al. 2004). It should be noted that our model
is consistent with most knockout studies of the DMRs (Ar-
ney 2003). Knockouts of H19 DMR or the Igf2 DMRs
may disrupt the looping structures. Upon maternal trans-
mision of a H19 DMR deletion, reduced H19 expression
accompanied by activated Igf2 expression was observed,
while upon paternal transmission of the deletion, reduced
Igf2 expression accompanied by activated H19 expression
was observed (Thorvaldsen et al. 1998). (It is noteworthy
that the expression levels of the activated genes were still
significantly lower than the wild type.) Thus, without the
H19 DMR, both the Igf2 and the H19 genes are not re-
stricted to a particular silent or active domain. Deletion of
the DMR1 of Igf2 leads to biallelic Igf2 expression upon
maternal transmission (Constancia et al. 2000), while
deletion of the DMR2 of Igf2 leads to transcriptional
down-regulation of Igf2 upon paternal transmission (Mur-
rell et al. 2001), which is consistent with DMR1 and
DMR2 contributing to the looping structure on the mater-
nal and paternal alleles, respectively.

A recent study shows that the paternal DMR2 is associ-
ated with the nucleolar membrane (Yusufzai et al. 2004).
Such interactions with subnuclear structures may well underlie the for-
mation or maintenance of functional chromatin loops. In
this way our observations would add to the growing body of
evidence suggesting that regulatory elements and gene
promoters in vertebrates are kept under tight control by
higher-order chromatin structures, especially loops (Chubb
and Bickmore 2003). The fascinating aspect of the obser-
vations on Igf2-H19 is that these higher-order structures
can be epigenetically regulated, thus providing simple epi-
genetic switches. It will be interesting to see whether other
imprinted loci, or other epigenetically regulated loci, such
as random monoallelically expressed genes (Chess 1998),
possess similar epigenetic switches.
THE Kcnq1ot1 LOCUS (IC2 DOMAIN)
Structure of the Locus and Regulatory Elements

The IC2 domain is located further telomeric from the IC1 domain but the two imprinted domains are closely linked (Fig. 1). Nevertheless, most of the genetic evidence available indicates that imprinting in the two domains is independently regulated. The IC2 domain contains the antisense RNA gene Kcnq1ot1, whose promoter region is currently defined as IC2. The Kcnq1ot1 gene is located within an intron of the Kcnq1 gene and its promoter overlaps with a DMR with germ line methylation arising from the oocyte (Engemann et al. 2000). The un-methylated paternal allele of Kcnq1ot1 is therefore transcribed while the maternal allele remains transcriptionally silent. All other genes in the ~800-kb domain surrounding IC2 are maternally expressed (or not imprinted; Fig. 1). The Kcnq1ot1 transcript partially overlaps with the large Kcnq1 gene, but this overlap probably does not extend to the Kcnq1 promoter region. A number of the genes in the IC2 domain are imprinted only in the placenta, attesting perhaps to the role in placental growth and function of a number of genes in the IC1 and IC2 domains. What is surprising is that most imprinted genes in the IC2 region do not have DMRs; this seems to be quite different from other larger imprinting clusters such as the PWS/AS region (Nicholls et al. 1998; A. Lewis et al., unpubl.).

The functional definition of the IC2 domain comes from knockout studies of the Kcnq1ot1 promoter region, both in cell lines and in mice. These show that if this region is removed from the paternal chromosome, the otherwise silent genes flanking it are now expressed (Horike et al. 2000; Fitzpatrick et al. 2002). This has been tested as far as Ascl2 on the centromeric side and Phlda2 on the telomeric side. By this criterion, the imprinted domain regulated by IC2 is at least 800 kb in size. Ascl2 is very likely the most centromeric imprinted gene in the domain, but the telomeric end has not been defined. It is unclear from these studies if it is the DNA sequence at the Kcnq1ot1 promoter (that was deleted in the IC2 knockout), the Kcnq1ot1 RNA, or the fact that the region is transcribed on the paternal chromosome that is responsible for cis-acting silencing. By transfection assays, the DNA segment itself appears to have methylation-sensitive silencer or insulator activity, depending on which cell line is used for the tests (Kanduri et al. 2002; Thakkar et al. 2003). By analogy with the Igf2r antisense gene Air, which is also paternally expressed, it is possible that the Kcnq1ot1 RNA (or the act of transcription) plays a role in silencing (Slosarek et al. 2002). This possibility is further strengthened by parallels with imprinted X-chromosome inactivation (below). What cis-acting sequences are responsible for imprinting and differential methylation of IC2 in the first place is unclear. A series of BAC and YAC transgenes containing IC2 and flanking sequences show that imprinting and the full tissue-specific pattern of expression (of Cdkn1c) depend upon the presence of remote sequence elements (John et al. 2001; F. Ceratto et al., unpubl.).

DNA Methylation Is Not Required for Maintenance of Imprinting: A Histone Methylation Imprint

Because of the relative scarcity of DMRs we were curious to see if DNA methylation was involved in regulating imprinting in the IC2 region, especially in the placenta. We used the Dnmt1 mutation, which removes the catalytic domain of Dnmt1 and results in a nonfunctional enzyme (Lei et al. 1996). Genomic methylation is dramatically reduced. Indeed, the maternal methylation of the Kcnq1ot1 promoter was completely lost in both Dnmt1−/− embryos and Dnmt1−/− oophoront (the precursor tissue of the placenta). This loss of DNA methylation causes a complete loss of imprinting at the IC2 cluster in the embryo. However, imprinted genes in the placenta that do not possess DMRs (such as Ascl2, Obph1, Kcnq1, etc.) do not lose imprinting (i.e., the paternal allele remains repressed) (A. Lewis et al., unpubl.). By contrast, the two genes with DMRs (Kcnq1ot1 and Cdkn1c) do lose imprinting and are now biallelically expressed. Thus the maintenance of placental imprinting in this region does not apparently require DNA methylation.

We therefore asked if the imprinted genes were marked by different types of epigenetic modifications. Chromatin immunoprecipitation was carried out in an allele-specific way using antibodies against acetyl and methyl modifications of histone tails. The Montpellier group (D. Umlauf and coworkers, unpubl.) show that this regional histone modification profile is only found in the placenta and in the embryo. In the embryo such allele-specific histone modifications are found only at confined regions comprising the DMRs. Other studies by D. Umlauf and coworkers (unpubl.) show that this regional histone methylation imprint is already present in ES cells, and thus may arise very early in development. It is then maintained in extraembryonic tissues, but apparently lost in embryonic ones.

The Repressive Histone Methylation Imprint Depends on IC2

We next asked if the histone methylation imprint depended on the IC2. Indeed, with paternal transmission of the IC2 deletion, histone modification differences as judged by allele-specific ChIP were eliminated at the several genes we analyzed. This indicates that the repressive histone methylation on the paternal chromosome is not established or is not maintained in mice carrying an IC2 deletion (A. Lewis et al., unpubl.). As stated before, it is
not yet clear whether the DNA element at IC2, Kcnq1ot1 transcription, or the Kcnq1ot1 transcript is responsible for attracting repressive histone modifications to the region in cis. Our favored model is that the transcript itself plays some role. Thus we envisage (Fig. 4) that the Kcnq1ot1 RNA is paternally expressed early in the preimplantation embryo. We then envisage that the noncoding Kcnq1ot1 RNA coats the IC2 region, and that this recruits, directly or indirectly, histone methyltransferases to the paternal chromosome. Indeed Umlauf et al. (unpubl.) show that the Eed/Ezh2 polycomb complex is recruited to the paternal chromosome in ES cells. This complex has K27 and some K9 methyltransferase activity. Other methyltransferases, such as perhaps G9a, might be recruited to methylate the K9 residue. Indeed, it is interesting that in the Eed knockout mouse some of the paternally silenced genes analyzed here are derepressed, but not all of them are (Mager et al. 2003). Therefore differential, and perhaps also additive, effects of the different repressive histone modifications might be imagined. In addition, it is likely that histone methylation and DNA methylation cooperate in silencing. For example, the silent copies of Kcnq1ot1 and of Cdkn1c (which possess DMRs) are considerably “more silent” than those of Kcnq1 or Cd81 (no DMRs), which show a bias only in parental expression. We have shown that the histone methylation imprint, once established, is independent of DNA methylation. But if our model is correct the IC2 domain may require the continued presence of the Kcnq1ot1 RNA to maintain imprinting. So in the Dnmt1–/– placenta, why does loss of methylation from the maternal Kcnq1ot1 promoter (causing derepression of maternal Kcnq1ot1 transcription) not result in silencing of the maternal chromosome? In heterozygous crosses between Dmnt1–/– animals, the maternal oocyte still contains large amounts of Dmnt1 protein, which may become depleted only toward the blastocyst stage (at which stage the histone methylation imprint is already established; DUmlauf et al., unpubl.). Thus activation of the maternal copy of Kcnq1ot1 may...
occur only around the blastocyst stage at which perhaps a "window of opportunity" for cis establishment of the histone methylation imprint is already closed.

Why is histone methylation apparently important in the placenta, but less so in the embryo, for imprinting? It is possible that imprinting coevolved with the placenta in mammalian radiation, and that initially imprinted genes acted primarily in the placenta to regulate supply of maternal nutrients to the fetus (Reik et al. 2003). We speculate that during this phase of imprinting evolution, histone methylation may have been involved in imprinting in marsupials, for example, the insulin-like growth factor 2 receptor gene is imprinted as it is in mice, but the DMR2 region, which in mice carries the DNA methylation imprint, is not methylated (Killian et al. 2000). Similarly, inactivated genes on the X chromosome are not methylated in female marsupials, but they are in female mice (Wakefield et al. 1997). Thus an ancestral imprinting mechanism may have been based on histone modifications and was perhaps initially limited to the placenta. Since we believe a mechanism based on only histone modifications is inact in the morula and early blastocyst, the majority of cells, including those in the inner cell mass, carry an inactive paternal X chromosome, and that silencing is erased (starting with down-regulation of Xist) in the ICM, followed by random X-inactivation at a slightly later stage in the epiblast (Mak et al. 2004, Okamoto et al. 2004). What is still debated is whether, in addition to early postzygotic action of Xist, the paternal X is already marked or preinactivated in the germ line, perhaps because of the X-inactivation event that occurs during spermatogenesis (Huynh and Lee 2003).

The Insulator-Loop Model and the Noncoding RNA Model: Application to Other Imprinted Regions

The noncoding RNA model is clearly applicable to other imprinted regions as well. First, there are over similarities with imprinted X-chromosome inactivation. The current model of imprinted X (in the mouse placenta) envisions that Xist is expressed from the paternal X as soon as the two-cell stage, and begins to coat the inactivating X soon after. Histone methylation recruitment also begins during preimplantation development, and so does gene inactivation along the paternal X chromosome, possibly in a gradient from the Xic locus (Huynh and Lee 2003); Mak et al. 2004; Okamoto et al. 2004). What is still debated is whether, in addition to early postzygotic action of Xist, the paternal X is already marked or preinactivated in the germ line, perhaps because of the X-inactivation event that occurs during spermatogenesis (Huynh and Lee 2003).

Regarding this uncertainty, it is generally thought that in the monula and early blastocyst, the majority of cells, including those in the inner cell mass, carry an inactive paternal X chromosome, and that this silencing is erased (starting with down-regulation of Xist) in the ICM, followed by random X-inactivation at a slightly later stage in the epiblast (Mak et al. 2004, Okamoto et al. 2004). We think that the noncoding RNA model is potentially applicable to a number of other imprinted loci, particularly those with paternal expression of noncoding antisense transcripts. The Igf2r locus is especially interesting since the Igf2r gene is overlapped by the noncoding antisense transcript Air, and the noncoding transcript or transcription is needed for cis inactivation of Igf2r as well as the two linked genes, Slc22a2 and Slc22a3, which are only imprinted in the placenta (and do not have DMRs; Stoddert et al. 2002). Thus our specific prediction for this locus would be that in the placenta there should be a histone methylation imprint in Slc22a2 and Slc22a3, and that genetic removal of DNA methylation should not lead to loss of imprinting of these Slc22a genes. The same model may well be applicable to other clusters with maternal germ line methylation and a role in placental growth and function. For example, the Snurf-Snrpn promoter transcribes a very long paternal transcript which may have a role in co-inactivation.

Conversely, we speculate that the looping-insulator model may be more widely applicable to clusters with paternal germ line methylation, such as Dlk1-Gt12, and Rasgrf1. Maternal deletion of the (unmethylated) DMR upstream of Gt12 leads to reactivation of the normally silent Dlk1 on the maternal chromosome, suggesting disruption of an insulator similar to that seen upstream of H19 (Lin et al. 2003). Rasgrf1 has a paternally methylated DMR with insulator activity (Yoon et al. 2002).

We must also consider the possibility that these two models are not necessarily mutually exclusive. Much of the regulatory machinery important for the epigenetic switch in the Igf2-H19 region is also present in the IC2 cluster. For instance the Kcnq1ot1 DMR has been shown to have methylation-sensitive insulator function in several somatic cell lines (Kanduri et al. 2002). Interestingly, in trophoblast-derived cells the DMR functions as a bidirectional silencer rather than a unidirectional insulator (Thakur et al. 2003). Perhaps these tissue-specific effects contribute to the differences in imprinting expression in embryonic and extraembryonic lineages. Perhaps the DMR region is also involved in a tissue-specific higher-order chromatin structure that isolates promoters and enhancers, or that isolates the IC2 cluster and its allele-specific histone modifications) from the surrounding loci. The Igf2-H19 cluster contains several noncoding transcripts including H19 itself. None appear to be as extensive as the Kcnq1ot1 transcript, which is at least 60 kb long, so a coating mechanism as described above seems unlikely. However, the process of transcription of these noncoding RNAs or the RNAs themselves may contribute to transcriptional, posttranscriptional, or RNAi-like mechanisms of gene regulation.

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REFERENCES

Barlow D.P., Stoger R., Herrmann B.G., Saito K., and Schweifer


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Kelsey G., and Reik W. 2001. An intragenic methylated re- 

Nicholls R.D., Saini S., and Horvath B. 1998. Imprinting in 

Okamoto I., Otte A.P., Allis C.D., Reineberg D., and Heard E. 
2004. Epigenetic dynamics of imprinted X inactivation during 

Palstra R.J., Tolhuis B., Splinter E., Nijmeijer R., Grosveld F., 
and de Laat W. 2003. The beta-globin nuclear compartment in 

Pari V., Murtino P., Kanduri C., Mattsson A., Lobanenkov V., 
Heuchel R., and Ohlsson R. 2003. The nucleotides responsi- 
bile for the direct physical contact between the chromatin ins- 
sulator protein CTCF and the H19 imprinting control region 
manifest parent of origin-specific long-distance insulation 
and methylation-free domains. Genes Dev. 17: 516.

Patterson G.P., de Krom M., de Boer E., Langeveld A., Imam 
Multiple interactions between regulatory regions are required 
to stabilize an active chromatin hub. Genes Dev. 18: 1495.

Reik W. and Walter J. 2001. Genomic imprinting: Parental in- 

Reik W., Constancia M., Fowden A., Anderson N., Dean W., 
of supply and demand for maternal nutrients in mammals by 
imprinted genes. J. Physiol. 547: 35.

Sauskar H., Jones P.A., Chariot J.R., Ferguson-Smith A.C., Bar- 
ton S.C., Reik W., and Surani M.A. 1992. Parental imprint- 
ing: Potentially active chromatin of the repressed maternal 
allele of the mouse insulin-like growth factor II (Igf2) gene. 
Genes Dev. 6: 1843.

maintains differential methylation at the Igf2/H19 locus. Nat. 
Genet. 33: 66.

Slutzik F. and Barlov D.P. 2002. The origins of genomic im- 

Slutzik F., Zwart R., and Barlov D.P. 2002. The non-coding 
Air RNA is required for silencing autosomal imprinted genes. Na- 
ture 415: 810.

Singer R., Kuchinka P., Liu C.G., Kafri T., Razin A., Cedar H., 
and Barlov D.P. 1993. Maternal-specific methylation of the 
imprinted mouse Igf2 locus identifies the expressed locus as 
carrying the imprinting signal. Cell 75: 61.

of reconstituted mouse eggs suggests imprinting of the 

Maternal-specific footprints at putative CTCF sites in the H19 
imprinting control region give evidence for insula- 

2004. Role of CTCF binding sites in the Igf2/H19 imprinting 

Thakar N., Kamishi M., Holmgren C., Mukhopadhyay R., and 
Kanduri C. 2003. Bidirectional silencing and DNA methyla- 
tion-sensitive methylation-spreading properties of the Kcnq1 
imprinting control region map to the same regions. J. Biol. 
Chem. 278: 9314.

Thorvaldsen J.L., Darnell K.L., and Bartolomei M.S. 1998. Dele- 
tion of the H19 differentially methylated domain results in 
loss of imprinted expression of H19 and Igf2. Genes Dev. 12: 
3693.

Tolhuis B., Palstra R.J., Splinter E., Grosveld F., and de Laat 
W. 2002. Looping and interaction between hypersensitive sites 

imprinting: Intricacies of epigenetic regulation in clusters. 

Wakefield M.J., Kerehane A.M., Turner B.M., and Groves J.A. 
1997. Histone underacetylation is an ancient component of 
Sci. 94: 9665.

Wehr M., Hagerger H., McPheeters A., Staud C., Reik W., Cathala 

Yoon B.J., Herman H., Sikora A., Smith L.T., Pluss C., and 
Sloboday P.D. 2002. Regulation of DNA methylation of Ras- 
g1. Nat. Genet. 30: 82.

Yusa T.M. and Felsenfeld G. 2004. The 5′-HS4 chicken 
beta-globin insulator is a CTCF-dependent nuclear matrix-as- 

CTCF tethers an insulator to subnuclear sites, suggesting 
shared insulator mechanisms across species. Mol. Cell 13: 
291.