

Chapter 2

Noncoding RNAs and Chromatin Modifications in the Developmental Control of Imprinted Genes

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Abstract In mammals, hundreds of protein-coding genes and regulatory noncoding RNAs (ncRNAs) are controlled by the epigenetic phenomenon of genomic imprinting. These unusual genes are organized in clusters in the genome, and their mono-allelic expression depends on whether the allele is inherited from the mother or from the father. The imprinted gene expression is mediated by essential regulatory sequence elements called “imprinting control regions” (ICRs), which carry mono-allelic DNA methylation marks. These germ line-derived imprints are maintained throughout development and after birth, a process which is linked consistently to specific chromatin modifications. The way ICRs mediate mono-allelic gene expression is tissue specific at many of the imprinted gene clusters. At several imprinted gene domains, the ICR expresses a long ncRNA that mediates chromatin repression *in cis*. At other imprinted domains, the ICR differentially structures higher-order chromatin that allows, or prevents, transcription of close-by genes. Here, I introduce the epigenetic phenomenon of genomic imprinting and discuss how long ncRNAs and chromatin contribute to its developmental regulation.

Keywords Genomic imprinting • Noncoding RNA • DNA methylation • Histone methylation • Chromatin

List of Abbreviations

5hm	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
BWS	Beckwith-Wiedemann syndrome
CpG	Cytosine-phosphate-guanine

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DMR	Differentially methylated region
gDMR	DMR with germ line-derived allele-specific DNA methylation
ICR	Imprinting control region
KMT	Lysine methyltransferase
lncRNA	Long noncoding RNA (more than 200 bases in length)
MBD	Methyl-CpG-binding domain
ncRNA	Noncoding RNA
PRC	Polycomb repressive complex
PWS	Prader-Willi syndrome
TNDM	Transient neonatal diabetes mellitus

2.1 Genomic Imprinting: An Epigenetic Phenomenon

Epigenetic processes contribute to development, homeostasis, and behavior by guiding the establishment and maintaining patterns of gene expression (Jaenisch and Bird 2003). Different epigenetic phenomena control transcription levels by conferring mono-allelic expression to specific genes or chromosomal domains. These gene dosage mechanisms include X chromosome inactivation in females and genomic imprinting (Lee and Bartolomei 2013), an essential epigenetic phenomenon that brings about mono-allelic gene expression entirely dependent on the parental origin of the gene (Ferguson-Smith 2011). Some imprinted genes are expressed from the paternally inherited allele only and repressed on the maternal chromosome. Others are expressed uniquely from the maternal chromosome.

In humans and mice, more than hundred protein-coding genes are controlled by genomic imprinting (Williamson et al. 2013; Morison et al. 2005). These exceptional genes have been shown to play diverse roles in growth and development, and many contribute to postnatal homeostasis and behavior as well (Peters 2014). During the last years, it has become apparent that hundreds of regulatory noncoding RNAs (ncRNAs), including regulatory microRNAs and long ncRNAs, are controlled by genomic imprinting as well (Girardot et al. 2012; Pauler et al. 2012). The biological functions of the imprinted ncRNAs remain poorly understood compared to what is known about protein-coding imprinted genes. Recent research shows that several imprinted long ncRNAs are involved in the control of imprinted gene expression during development. Here, I review the contribution of imprinted ncRNAs and covalent chromatin modifications in the developmental control of imprinted gene expression.

A mechanistically important feature of mammalian imprinted genes is that the large majority are organized in evolutionarily conserved clusters, which constitute chromosomal domains of up to several mega-bases (Mb) in size (Kelsey and Feil 2013). At each of these domains, the imprinted, mono-allelic expression of genes is controlled by a so-called imprinting control region (ICR). Briefly, ICRs are essential regulatory DNA elements of up to several kilobases in size, which are rich in CpG dinucleotides, and are marked by germ line-derived 5-methylcytosine (5mC)

methylation on one of the two parental copies only. Although each ICR consists of unique DNA sequences, a unifying hallmark of ICRs is that they all carry parent-of-origin-specific DNA methylation. Furthermore, at all ICRs, the mono-allelic DNA methylation is maintained throughout development, including through the critical pre- and peri-implantation stages of development (Kota and Feil 2010; Smallwood and Kelsey 2012). This behavior contrasts to that of the bulk of DNA methylation in the early embryo, which undergoes global waves of DNA demethylation and DNA de novo methylation (Feil 2009a; Reik et al. 2001). The exceptional somatic maintenance of the differential DNA methylation at ICRs involves, besides the DNA methylation itself, different nuclear proteins and covalent histone modifications, particularly histone lysine methylation (Kacem and Feil 2009; Kelsey and Feil 2013).

In contrast to the mechanisms that ensure somatic maintenance, which are highly similar between ICRs, the way these control regions bring about mono-allelic expression of genes differs between them. At many of the known domains, imprinted gene expression occurs in some but not in other tissues (Peters 2014). This indicates the ICRs at these domains are “read” in a tissue-specific manner. We are only at the beginning of understanding the tissue-specific effects of ICRs. Nevertheless, There is evidence for the involvement of covalent chromatin modifications, particularly histone lysine methylation, and a role for higher-order chromatin structuration (Kacem and Feil 2009). At several imprinted loci, interestingly, the tissue-specific action of the ICR also requires the expression of imprinted long ncRNAs (lncRNAs), which have diverse effects on chromatin and gene regulation. These novel mechanistic insights are presented and discussed in further detail below.

2.2 Chromatin Features that Control Germ Line-Derived DNA Methylation

2.2.1 *Acquisition of Imprinted DNA Methylation*

The specificity of the methylation imprints at ICRs is linked to their establishment in either the male or the female germ line. After fertilization, the germ line-derived DNA methylation is somatically maintained throughout development. Most ICRs are methylated on the maternally inherited allele, carrying a methylation mark that is acquired late in oogenesis, during the final maturation and growth of oocytes. So far, only three ICRs have been reported to be “paternally methylated” in mice and humans, with their allelic DNA methylation imprint originating from sperm. At these three regions, the paternal imprint acquisition occurs during the fetal, premeiotic stages of spermatogenesis (Kota and Feil 2010).

Despite the distinctly different timing of imprint establishment in female versus male germ cells, in the adult and fetus, respectively, the de novo DNA methylation

machinery is the same in both the germ lines. In both, there is a strict requirement for the de novo DNA methyltransferase DNMT3A and that of a non-catalytically active partner protein, called DNMT3-like (DNMT3L) (Kelsey and Feil 2013; Kota and Feil 2010). What determines that the DNMT3A/DNMT3L protein complex is brought to one set of ICR regions in one germ line and to another set in the opposite germ line? Insights into this key question have emerged during the last years. Particularly, several studies have shown that transcription through the ICR sequences is linked to the acquisition of DNA methylation by DNMT3A and could thus be essential to the process (Chotalia et al. 2009; Henckel et al. 2012). How chromatin is organized and which covalent histone modifications it carries could be relevant for imprint acquisition as well. Particularly, a lack of histone H3 methylation at lysine 4 is important to provide the methylation machinery (DNMT3/DNMT3L) access to its target regions (Ciccone et al. 2009; Feil 2009b; Henckel et al. 2012; Ooi et al. 2007). These novel insights into the specificity of imprint acquisition in the female and male germ lines are discussed in detail in several recent reviews (Duffie and Bourc'h 2013; Kelsey and Feil 2013; Kota and Feil 2010) and will not be considered further in this chapter.

2.2.2 *Somatic Maintenance of the Methylated Alleles of ICRs*

Another, equally important determinant of imprint specificity is the somatic maintenance of the differential DNA methylation status of ICRs after fertilization and during early development. Through genome-wide methylation studies, it has become clear that hundreds of promoter/CpG island regions become methylated in germ cells, particularly in the female germ line (Kobayashi et al. 2012; Smallwood et al. 2011). However, this differential methylation is maintained at only some of these regions in the preimplantation embryo, and these exceptional regions include all the known ICRs (Duffie and Bourc'h 2013; Reik et al. 2001). Thus, the specificity of imprints is determined to a great extent by their exceptional somatic maintenance in the early embryo.

The stable epigenetic maintenance of ICRs in the embryo is linked to patterns of histone lysine and arginine methylation that are different between the parental chromosomes (Kacem and Feil 2009). This consistent link suggests that histone methylation contributes to the maintenance of the differential DNA methylation at ICRs. During the last years, several of the responsible histone-modifying enzymes have been identified, and this has allowed functional studies to be performed (Kelsey and Feil 2013).

Particularly, chromatin associated with the DNA-methylated alleles of ICRs is consistently enriched in histone H3 lysine 64 trimethylation (H3K64me3), H3 lysine 9 trimethylation (H3K9me3), H4 lysine 20 trimethylation (H4K20me3), and H4 arginine 3 symmetrical dimethylation (H4R3me2s) and is bound by the heterochromatin protein 1 gamma (HP1 γ) as well (Delaval et al. 2007; Di Cerbo et al. 2014; Girardot et al. 2014; Pannetier et al. 2008). In addition, the methylated

alleles of ICRs are enriched in the noncanonical histone H3 variant H3.3, which is incorporated replication independently by the ATRX/DAXX histone chaperone complex (Voon et al. 2015). These “repressive chromatin” features are similar to those found at the constitutively maintained heterochromatin of peri-centromeric regions, but to which extent they contribute to the maintenance of the DNA methylation remains unclear. Nevertheless, studies on embryonic stem (ES) cells have described small losses of DNA methylation due to depletion of specific lysine methyltransferases (KMTs), including the H3 lysine 9-modifying enzymes G9A and ESET/SETDB1 (Girardot et al. 2014; Leung et al. 2014; Quenneville et al. 2011). Whether these findings reflect the *in vivo* situation in the embryo is not at all clear. In the embryo, for instance, the absence of G9A does not result in loss of DNA methylation at ICRs (Wagschal et al. 2008; Xin et al. 2003; Auclair et al. 2015).

DNMT1 is the maintenance DNA methyltransferase which ensures that DNA methylation imprints are faithfully maintained in the embryo (Hirasawa et al. 2008). Several other proteins are involved in the process as well. Genetic studies on imprinting-related diseases in humans (Hirasawa and Feil 2010) pinpointed several nuclear proteins that contribute to the somatic maintenance of imprints by DNMT1 in the early embryo (Table 2.1). One of these is ZFP57 (Li et al. 2008), a KRAB domain zinc finger protein which recruits KAP1 (also called TRIM28 or TIF1B) to the chromatin. The KAP1 “platform protein” in turn, mediates recruitment of histone-modifying enzymes, including the H3 lysine 9-specific ESET/SETDB1, which regulates H3K9me3 at ICRs (Hirasawa and Feil 2008). Significantly, ZFP57 binds to methylated DNA sequences only and recognizes a specific sequence motif (“TGCCGC”) found at most ICRs and at some other, differentially methylated regions (DMRs) as well (Quenneville et al. 2011; Strogantsev et al. 2015). *ZFP57* mutations are associated with the childhood disorder “transient neonatal diabetes mellitus” (TNDM). In TNDM children that carry *ZFP57* mutations, the aberrant *ZFP57* expression causes a loss of DNA methylation at the imprinted *PLAGL1* transcription factor gene, and it is this epigenetic change which is causally involved in the disease phenotype (Mackay et al. 2008). In some TNDM patients with inactivating *ZFP57* mutations, interestingly, the DNA methylation loss affects several other ICRs as well, indicating a considerable phenotypic heterogeneity. Mouse studies have confirmed that the zinc finger protein ZFP57 is essential for the maintenance of imprints during development at multiple ICRs (Quenneville et al. 2011).

The first cell divisions of development are particularly critical for imprint maintenance, and several maternal (i.e., oocyte-derived) proteins were shown to be essential at these early preimplantation stages. One of these is DPPA3 (also called Stella or PGC7), a protein present at high levels in oocytes which persists in the early embryo. This chromatin-associated protein helps to maintain DNA methylation on the maternal set of chromosomes in the post-zygotic embryo, including at several maternal ICRs (Nakamura et al. 2007). Unexpectedly, given that this is a maternally derived protein factor, it was found that DPPA3 also contributes to the early embryonic maintenance of methylation at two of the paternal ICRs. DPPA3

Table 2.1 Somatic maintenance of differential DNA methylation at imprinting control regions (ICRS) in mammals

Name	Characteristic(s)	Phenotype due to loss of expression or to knockdown, in somatic cells/embryos	Reference(s)
(Candidate) factors that prevent de novo DNA methylation at ICRs			
OCT4 (POU5F1)	Pluripotency transcription factor	Binding-site mutations induce hypermethylation at the ICR of <i>IGF2-H19</i> locus	Abi Habib et al. (2014); Hori et al. (2012)
SOX2	Pluripotency transcription factor	Binding-site mutations induce hypermethylation at the ICR of the <i>IGF2-H19</i> locus	Abi Habib et al. (2014); Hori et al. (2012)
CTCF	Zinc finger protein involved in higher-order chromatin structuration	Hypermethylation at the ICR of the <i>Igf2-H19</i> domain	Schoenherr et al. (2003)
ZFP42	Zinc finger protein associated with pluripotency	Hypermethylation at the ICRs of the <i>Peg3</i> and <i>Gnas</i> domains	Kim et al. (2011)
R-loops	RNA-DNA structures found on the unmethylated alleles of ICRs	Possible protection against de novo DNA methylation at ICRs (<i>Igf2r</i> and <i>Snrpn</i> loci)	GINNO et al. (2012)
Factors that maintain DNA methylation at ICRs			
DNMT3A	De novo DNA methyltransferase	Non-maintenance of DNA methylation due to a lack of de novo methylation	da Rocha et al. (2007)
DNMT1	Maintenance DNA methyltransferase	Loss of DNA methylation, loss of imprinted expression at several genes	Hirasawa et al. (2008)
ZFP57	KRAB domain zinc finger protein	Partial loss of DNA methylation at several ICRs (<i>PLAGL1</i> , <i>GRB10</i> , and <i>PEG3</i>)	Li et al. (2008); Mackay et al. (2008); Strogantsev et al. (2015)
MBD3	Methyl-CpG-binding domain protein 3	Partial loss of DNA methylation at the ICR of the <i>Igf2-H19</i> locus	Reese et al. (2007)
MTA2	Metastasis tumor antigen 2	Partial loss of DNA methylation at the ICRs of the <i>Peg3</i> and <i>Igf2-H19</i> domains	Ma et al. (2010)
KAP1	KRAB-associated protein 1	Partial loss of DNA methylation at several ICRs (<i>Igf2-H19</i> and <i>Snrpn</i> loci)	Giovarelli et al. (2014)
UHRF1 (NP95)	Binds to hemimethylated DNA after replication; proposed to recruit DNMT1	Partial loss of DNA methylation at the ICRs of the <i>Igf2-H19</i> , <i>Kcnq1</i> , and <i>Dlk1-Dio3</i> domains	Di Cerbo et al. (2014)
H1 histones	Linker histones	Triple knockout (<i>H1c</i> , <i>H1d</i> , <i>H1e</i>): hypomethylation at the ICRs of the <i>Igf2-H19</i> and <i>Dlk1-Dio3</i> domains	Yang et al. (2013)

(continued)

Table 2.1 (continued)

Name	Characteristic(s)	Phenotype due to loss of expression or to knockdown, in somatic cells/embryos	Reference(s)
RBBP1/ RBBP1L1	Retinoblastoma (Rb)-binding proteins	Combined knockout: loss of ICR DNA methylation at the PWS (<i>Snrpn</i>) domain	Wu et al. (2006)
NLRP2	Cytoplasmic caterpillar family protein	(Somatic?) loss of DNA methylation at the ICR of the <i>KCNQ1</i> domain	Meyer et al. (2009)
DPPA3 (PGC7/ Stella)	Methylated histone (H3K9me2)-binding protein	Partial loss of DNA methylation at several ICRs	Nakamura et al. (2007)

protects against DNA demethylation by inhibiting the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) in the zygote (Wossidlo et al. 2011). How DPPA3 is targeted to the specific loci it protects, including ICRs, is not clear. The protein was shown to recognize chromatin marked by H3 lysine 9 dimethylation (H3K9me2), a covalent modification which is present on the DNA-methylated allele of several ICRs (Nakamura et al. 2012). At later developmental stages, also the embryonically expressed form of DPPA3 is thought to contribute to imprint maintenance, particularly in the pluripotent cells of the preimplantation embryo (Xu et al. 2015).

Other proteins that contribute to the maintenance of methylation imprints include MBD3, methyl-CpG-binding domain protein 3. MBD3 is among several proteins that associate specifically with the methylated alleles of ICRs. It is part of the “nucleosome remodeling and deacetylase” (NuRD) complex, a large repressive complex which also includes the protein metastasis tumor antigen 2 (MTA2). Knockdown of either *Mta2* or *Mbd3* led to a partial loss of CpG methylation at the paternally methylated ICR of the *Igf2-H19* locus in mouse embryos but not at several other ICRs that were analyzed (Ma et al. 2010; Reese et al. 2007). This shows that NuRD complex components are involved in imprint maintenance, at least at this single ICR.

Retinoblastoma-binding protein 1 (RBBP1, also named ARID4A) binds with low affinity to both H3K9me3 and H4K20me3 (Gong et al. 2012). This nuclear protein was discovered to be involved in the control of gene expression at the imprinted domain associated with the neurobehavioral disorder, Prader-Willi syndrome (PWS). Targeted deletion of *Rbbp1* in the mouse, together with that of the related gene *Rbbp1-like 1* (*Rbbp1L1*), gave reduced levels of H3K9me3 and H4K20me3 at the ICR controlling the PWS domain, and this correlated with a partial loss of DNA methylation (Wu et al. 2006). This important finding highlights that “readers” of histone lysine methylation contribute to the somatic maintenance of methylation imprints as well, at least at the ICR of the PWS domain.

2.2.3 Somatic Maintenance of the Unmethylated Alleles of ICRs

Recent mouse studies emphasize the importance of protecting the non-methylated allele of DMRs against acquisition of de novo DNA methylation during development (Proudhon et al. 2012). Different nuclear proteins contribute to this essential process (see Table 2.1). The zinc finger proteins ZFP42 (Kim et al. 2011) and “CCCTC-binding factor” (CTCF) are among these protective proteins. CTCF has been studied in most detail and is an essential architectural protein involved in chromatin looping and long-range chromatin interactions. It binds with high affinity to the non-methylated allele of the ICR of the *Igf2-H19* imprinted domain. Deletion of all CTCF-binding sites at the ICR abrogates CTCF binding and leads to acquisition of aberrant DNA methylation at the ICR’s normally unmethylated maternal allele (Engel et al. 2006; Schoenherr et al. 2003).

Interestingly, the *H19-Igf2* ICR also comprises multiple binding sites for the pluripotency-associated transcription factors SOX2 and OCT4 (also called POU5F1). Mutations of these binding sites are linked to aberrant gains of DNA methylation in humans, and the resulting biallelic ICR methylation gives rise to biallelic expression of the growth factor *IGF2* and, hence, to the fetal overgrowth disorder Beckwith-Wiedemann syndrome (BWS) (Abi Habib et al. 2014). This recent finding indicates that pluripotency factors protect the unmethylated allele of this ICR against de novo methylation in the early embryo. Future studies should unravel whether pluripotency transcription factors such as OCT4 and SOX2 protect the unmethylated alleles of other ICRs as well.

A consistent feature of chromatin associated with the unmethylated alleles of ICRs is that histones H3 and H4 are acetylated at lysine residues and that there is a local enrichment of H3 lysine 4 dimethylation and/or trimethylation (H3K4me2/H3K4me3) (Delaval et al. 2007). Through biochemical studies, this H3K4 methylation was shown to prevent the DNMT3A/DNMT3L complex to bind to the chromatin, and through this mechanism, it protects against de novo DNA methylation (Feil 2009b; Guo et al. 2015; Ooi et al. 2007).

Most ICRs comprise CpG island promoters, which are transcriptionally active on the unmethylated parental allele only. This likely explains why, consistently, there is an “active chromatin” configuration characterized by histone acetylation and H3K4me2/H3K4me3 on the unmethylated alleles of ICRs. Linked to transcription events also, a recent study shows that there is formation of R-loops on the unmethylated alleles of ICRs as well (Ginno et al. 2012). These double-stranded RNA-DNA structures form on the transcribed DNA strand, and they have been observed most strongly in embryonic stem (ES) cells. In vitro studies have suggested that these R-loops protect the unmethylated allele of ICRs against acquisition of de novo DNA methylation in the early embryo (Ginno et al. 2012). Thus, several transcription-related mechanisms protect the unmethylated alleles of ICRs against DNA methylation in the embryo.

2.3 Higher-Order Structuration of Chromatin at Imprinted Gene Loci

The allelic methylation imprints and their associated chromatin features make the two parental copies of ICRs nonequivalent. These epigenetic modifications create a functional difference, and this leads to parental allele-specific expression of close-by genes in the embryo, a process which is often lineage- or tissue-specific manner. However, the mechanisms at ICRs that mediate imprinted gene expression differ between domains. One of the earliest unraveled scenarios is the formation of allelic chromatin loops by the ICR and the demonstration that this process depends on the ICR's allelic methylation status.

The best-studied example of ICR-mediated loop formation is the *Igf2-H19* locus on mouse chromosome 7 (human chromosome 11q15). On its unmethylated (maternal) allele, the domain's ICR confers a chromatin loop that prevents the distally located insulin-like growth factor-2 (*Igf2*) gene to interact with proximally located enhancers. This so-called boundary function requires recruitment of the 11-zinc finger protein CTCF to the ICR, which comprises multiple binding motifs. The CTCF binding triggers the subsequent recruitment of cohesin proteins. The constituted CTCF-cohesin complexes induce higher-order loop structures on the maternal chromosome that allow expression of the *H19* gene. On the methylated paternal chromosome, there is no CTCF-cohesin complex formation, and this facilitates a different higher-order chromatin organizing which allows expression of *Igf2* in *cis* (Kurukuti et al. 2006; Murrell et al. 2004).

CTCF and cohesins are broadly expressed in the embryo. Unexpectedly, however, the *Igf2* gene does not display paternal allele-specific expression in all the tissues. For instance, there is biallelic (non-imprinted) *Igf2* expression in the leptomeninges and the choroid plexus of the brain (Feil et al. 1994). Most likely, tissue-specific factors influence the ICR's boundary function and/or its effects on close-by genes, and this differential regulation may include yet unknown factors that activate alternative enhancers in the specific tissues that show biallelic *Igf2* expression.

Imprinted gene expression requires CTCF-mediated chromatin looping at other imprinted domains as well (Franco et al. 2014). One example is provided by an imprinted locus comprising the paternally expressed gene 13 (*Peg13*) and the potassium channel gene *Kcnk9* on mouse chromosome 15. These genes show imprinted expression in the brain, both in mice and humans, with *Peg13* expressed from the paternal and *Kcnk9* from the maternal chromosome. *Peg13* comprises the one germ line DMR (gDMR) of the locus which is methylated on the maternally inherited chromosome. The unmethylated paternal copy of this putative ICR is bound by cohesins and CTCF. Chromatin conformation capture ("3C") studies have been performed on the locus, and the obtained data suggest that the CTCF/cohesin binding to the gDMR prevents an enhancer region to loop onto the *Kcnk9* gene. This model explains the lack of *Kcnk9* expression on the paternal chromosome. On the maternal chromosome, the gDMR is fully methylated and is therefore not bound

by CTCF/cohesin, and on this parental chromosome, enhancer-promoter interactions that activate *Kcnk9* are readily detected by the 3C technology (Court et al. 2014). It has been hypothesized that the CTCF/cohesin-mediated imprinted expression at the *Peg13-Kcnk9* domain could be relevant to human disease, since this chromosomal region is genetically linked to an autosomal recessive dysmorphism and mental retardation syndrome (Court et al. 2014).

A newly emerged insight is that at some imprinted domains, chromatin looping somehow depends on imprinted long noncoding RNAs (lncRNAs) as well (Franco et al. 2014). The imprinted *Kcnq1* domain on mouse chromosome 7 is controlled by a maternally methylated ICR. This ICR is located in an intron of the *Kcnq1* gene, one of the imprinted genes of the 750-kb *Kcnq1* domain. Long-range chromatin interactions at this domain require an lncRNA called *Kcnq1ot1*, which is expressed from the unmethylated copy of the ICR. Recent research indicates that the *Kcnq1ot1* lncRNA stabilizes particular long-distance chromatin interactions between the ICR and the *Kcnq1* gene promoter. This higher-order structuration is CTCF dependent (the ICR comprises two CTCF-binding sites) and contributes to the silencing of the *Kcnq1* gene on the maternal chromosome (Zhang et al. 2014; Schultz et al. 2015).

2.4 Long Noncoding RNAs that Mediate Imprinted Gene Silencing

The *Kcnq1* domain is not the only imprinted gene cluster that expresses a regulatory lncRNA. In fact, most imprinted domains express at least one imprinted lncRNA, transcription of which, or the RNA itself, is thought to control close-by genes (Pauler et al. 2012). Recent research indicates that imprinted lncRNAs affect gene expression not just in *cis* but can in some cases have effects in *trans* as well (Kaneko et al. 2014; Stelzer et al. 2014). This exciting insight consolidates the earlier idea of cross talk between imprinted genes, a group of genes which are co-regulated in their expression levels in individual tissues in different studies (Al Adhami et al. 2015; Cattanaach et al. 2004; Varrault et al. 2006).

2.4.1 lncRNA-Mediated Gene Repression in Cis

The imprinted Igf2-receptor (*Igf2r*) locus on mouse chromosome 17 is controlled by a maternally methylated ICR. This intragenic ICR is located in the second intron of the *Igf2r* gene itself. It comprises the promoter of an lncRNA called *Airn*, which is expressed from the paternal allele in the antisense orientation to *Igf2r* (Latos et al. 2012; Pauler et al. 2012). Knock-in studies in the mouse have inserted premature polyadenylation sites, and these transgenic explorations have shown

that transcription of the 118-kb *Airn* lncRNA is required for the allelic repression of *Igf2r* on the paternal chromosome. In addition, the lncRNA itself is required to allelically repress the cation transporter genes *Slc22a2* and *Slc22a3* that are imprinted in the extraembryonic lineages only (Sleutels et al. 2002).

Similar functional studies have been performed on the *Kcnq1* domain, to prevent transcription of the full-length lncRNA *Kcnq1ot1* through insertion of a premature polyadenylation signal (Pandey et al. 2008). This research established that also the lncRNA *Kcnq1ot1* mediates gene repression in *cis* (Pandey et al. 2008). Several of the *Kcnq1ot1*-controlled genes at the domain are imprinted in the extraembryonic lineages only (Umlauf et al. 2004). The latter finding provides an additional parallel with the imprinted *Igf2r* locus in the mouse.

Through which mechanisms do imprinted lncRNAs exert their repressive effects at their imprinted domains? It has been a challenge to explore this question, since it has not been easy to distinguish between effects due to the transcription of the lncRNA gene and possible effects of the lncRNA itself (Bassett et al. 2014; Pauler et al. 2012). Nevertheless, there is now ample evidence that the *Kcnq1ot1* and *Airn* lncRNAs are both directly involved in chromatin repression in *cis* in the extraembryonic lineage. In the placenta, this mechanism involves repressive histone H3 lysine methylation and histone H2A mono-ubiquitination (Lewis et al. 2004; Nagano et al. 2008; Pandey et al. 2008; Terranova et al. 2008; Umlauf et al. 2004).

In the embryo proper, things are mechanistically different. Here, transcription of *Airn* lncRNA is important for bringing about repression at the promoter of the host *Igf2r* gene. Extensive gene targeting and molecular studies (Latos et al. 2012) strongly suggest that this occurs through a transcriptional interference mechanism, rather than involving *Airn* itself. The *Slc22a2* and *Slc22a3* genes are not imprinted in the embryo itself, which agrees with the idea that *Airn* has a different role in the embryo compared to the extraembryonic lineage. Similarly, at the *Kcnq1* imprinted domain, the embryo shows a much more restricted pattern of imprinted gene expression compared to the extraembryonic membranes. Specifically, the genes that are repressed in *cis* by the lncRNA *Kcnq1ot1* in the placenta do not show evidence for imprinted expression in the embryonic tissues (Umlauf et al. 2004).

2.4.2 Histone Methylation Controls Placenta-Specific Imprinting

At the *Kcnq1* domain, the placental genes controlled by *Kcnq1ot1* lncRNA are located in the proximal and distal parts of the domain. The paternal allele-specific silencing of these genes in the mouse placenta is associated with H3 lysine 27 trimethylation (H3K27me3), H2A lysine-119 mono-ubiquitination (H2AK119ub), and H3K9me2 (Terranova et al. 2008; Umlauf et al. 2004). These covalent histone modifications are enriched on the chromatin at the distal and proximal parts of the imprinted domain, specifically in the placenta. The silenced

promoters of the placental genes do not acquire repressive DNA methylation, though, and the somatic maintenance of the chromatin-mediated gene repression has been shown to be entirely independent of regulators of DNA methylation (Lewis et al. 2004; Sleutels et al. 2002; Tanaka et al. 1999).

Whereas the repressive H2AK119ub is controlled by the polycomb group protein RING1B, which is part of the polycomb repressive complex 1 (PRC1), the H3K27me3 is controlled by EZH2, the catalytic unit of the PRC2 complex. Similarly as observed for *Hox* genes in embryonic cells (Eskeland et al. 2010), the RING1B-mediated H2AK119ub at the *Kcnq1* domain induces a more compacted chromatin state, measurable by DNA fluorescence in situ hybridization (FISH) with combinations of DNA probes at different positions in the domain (Terranova et al. 2008). It remains to be discovered how precisely the lncRNA facilitates the PRC1 and PRC2 complexes to bind to specific target loci and why this occurs in a tissue-specific manner while these PRC complexes themselves are ubiquitously expressed.

The repressive H3K9me2 at the *Kcnq1* domain is not mechanistically linked to the recruitment of polycomb group proteins but, rather, is controlled by the lysine methyltransferase (KMT) G9A (Wagschal et al. 2008). The recruitment of this specific KMT to the placental genes depends on the expression of the full-length *Kcnq1ot1* ncRNA as well (Pandey et al. 2008). Concordantly, RNA/DNA FISH studies have suggested that in placental cells the *Kcnq1ot1* lncRNA co-localizes with the placental gene loci at which it controls imprinted expression (Redrup et al. 2009). How this lncRNA mediates the recruitment and/or the activity of PRC1 and PRC2 complexes, and that of the KMT G9A, onto specific target genes remains elusive. This must be a tissue-specific process, however, confined to the extra-embryonic lineage, despite the ubiquitous expression of the *Kcnq1ot1* itself (Umlauf et al. 2004; Wagschal et al. 2008). Recent biochemical studies show that EZH2, the PRC2 catalytic protein that trimethylates H3 lysine 27, can bind to lncRNAs including *Kcnq1ot1* in vitro (Kaneko et al. 2014; Zhao et al. 2010). It remains to be determined in vivo whether lncRNA-protein interactions guide PRCs to the placental target genes or whether, alternatively, the lncRNAs alter the functionality of PRC complexes that are already bound to the chromatin.

The KMT G9A was found to be essential for the allelic repression of *Slc22a3*, one of the two placentally expressed imprinted genes of the *Igf2r* domain. G9A is recruited by the *Airn* lncRNA to the G9A promoter region in mid-gestation placenta, and this brings about the repressive H3K9 methylation (Nagano et al. 2008; Sleutels et al. 2002). Biotin-based biochemical labeling and purification studies have indicated that *Airn* physically associates with *Slc22a3*, which is located hundreds of kilobases (kb) away from the *Airn* transcription site (Nagano et al. 2008). Again here, how precisely the targeting of the lncRNA is controlled and what determines its pronounced tissue specificity remain to be discovered.

A third imprinted gene domain with G9A- and PRC2-dependent placenta-specific imprinting is the *Peg10/Sgce* locus on mouse chromosome 6 (Monk et al. 2008), but this domain has not been studied in as much detail. The one gene

that shows placenta-specific imprinting at this domain is the transcription factor gene *Tfpi2* (Monk et al. 2008).

Targeting studies in the mouse have confirmed that repressive histone methylation indeed contributes to imprinted gene regulation in the placenta. In G9A-deficient placentae, for instance, there is a partial relaxation of imprinting at several placental genes (*Ascl2*, *Cd81*, and *Obpl5*) of the *Kcnq1* domain, at the *Slc22a3* gene of the *Igf2r* domain, and at the *Tfpi2* gene of the *Peg10/Sgce* locus. Interestingly, the extent of this “loss of imprinting” varies between individual G9A-deficient placentae (Wagschal et al. 2008). The latter finding agrees with the fact that G9A and/or the H3K9me2 it mediates represent not the only repressive “layer” involved in the allelic repression of these genes (Wagschal et al. 2008).

In EED-deficient mouse conceptuses, which completely lack the PRC2 complex, partial loss of imprinting was reported at the placentally expressed *Ascl2* gene of the *Kcnq1* domain (Mager et al. 2003), and a similar phenotype is apparent at the *Tfpi2* gene of the *Peg10/Sgce* locus (Monk et al. 2008).

Which tissue-specific factors determine the specificity of the placental imprinting remains to be discovered. To address this key question, different groups have explored whether the tissue specificity of imprinting is conserved in humans. Similarly as in mouse placenta, the *TFPI2* gene at the *PEG10/SGCE* domain is expressed from the maternal chromosome only in human placenta (Monk et al. 2008). In mid-gestation human placenta, there is also imprinted expression of the *SLC22A2* and *SLC22A3* genes of the *IGF2R* locus, as in the mouse. This imprinted expression is polymorphic, however, with only some of the placentae displaying mono-allelic imprinted expression, and this correlated with expression of the lncRNA AIRN (Monk et al. 2006).

The placental genes *ASCL2*, *CD81*, *OSBPL5*, and *NAPIL4* at the *KCNQ1* domain, contrary to in the mouse, are expressed from both the parental chromosomes in human placenta (Monk et al. 2006). This biallelic expression in humans may imply that specific factors involved in the placental imprinting in mice are missing in human placenta. The lncRNA *Kcnq1ot1* itself is similarly expressed in the two species, however, and other factors must be functionally divergent between the two species.

Other imprinted lncRNAs that are thought to repress chromatin in *cis* include the Nespas lncRNA at the *Gnas* imprinted locus and the Gtl2 (also called Meg3) lncRNA at the *Dlk1-Dio3* imprinted domain (Williamson et al. 2013{da Rocha, 2007 #619}). The *Dlk1-Dio3* imprinted domain on mouse chromosome 12 is also particularly interesting as it expresses many ncRNAs from the maternally inherited chromosome only, including some 50 imprinted microRNAs, many C/D box snoRNAs, and long ncRNAs (da Rocha et al. 2008).

During the last years, the lncRNA Gtl2/Meg3 has attracted much attention. This nuclear lncRNA was reported to interact in vitro with the EZH2 protein of the PRC2 complex {Kaneko, 2014 #504}(Zhou et al. 2010). In addition, in vivo, it remains locally present and accumulates after transcription on the locus. It is visible by RNA FISH as an RNA “cloud” on the maternal copy of the *Dlk1-Dio3* domain in mouse embryonic stem (ES) cells and iPS cells (Kota et al. 2014). The possible association

of this lncRNA with the PRC2 complex, observed *in vitro*, could be relevant given that several of the protein-coding genes of this imprinted domain are marked by repressive H3K27me3 in genome-wide ChIP-seq studies. This lncRNA could therefore contribute to PRC2-mediated repression of protein-coding genes in *cis* on the maternal chromosome, which would agree with its developmental expression pattern (da Rocha et al. 2007). Additionally, *Gtl2* lncRNA could possibly also have effects in *trans* given that its perturbed expression in iPS cells affected the expression of genes elsewhere in the genome as well, although it is not excluded that these effects are indirect (Kaneko et al. 2014). Gene-targeting studies in the mouse have disrupted *Gtl2* expression to directly test the *in vivo* function of this lncRNA (Zhou et al. 2010{Takahashi, 2009 #621}). In these studies, it has been difficult however to discern effects due to the loss of *Gtl2* expression and concomitant DNA methylation changes at the ICR of the imprinted domain. In both these studies, however, loss of *Gtl2* expression correlates with a loss of imprinting at *Dlk1*, which is one of the protein-coding genes of the domain.

In humans, the *DLK1-DIO3* imprinted domain is located on chromosome 14q32.2 and is associated with rare disease syndromes affecting skeletal development and mental functions (Girardot et al. 2013; Kagami et al. 2008). Different studies have explored the occurrence of micro-deletions in patients. Recently, patients were identified with micro-deletions affecting the *GTL2 (MEG3)* promoter region, which leads to a complete loss of *Gtl2* RNA and the other maternal ncRNAs of the domain (Beygo et al. 2014). Interestingly, this loss of paternal ncRNA expression is associated with a loss of imprinting of *DLK1* (biallelic expression), a finding which agrees with the hypothesis that lncRNA could be involved in gene repression in *cis*. Further research is required to ascertain the role of the *Gtl2* lncRNA in the repression in *cis* of protein-coding genes at the domain and to unravel the precise underlying mechanism.

2.4.3 *Imprinted LncRNAs with Effects in Trans on Other Imprinted Gene Loci*

Imprinted lncRNAs also exert repressive effects in *trans*, at imprinted gene loci located elsewhere in the genome. This was discovered recently for an lncRNA at the imprinted PWS domain, called IPW, which is transcribed from the paternally inherited chromosome only in different tissues, particularly in brain (Stelzer et al. 2014). In human-induced pluripotent stem (iPS) cell clones, its knockdown led to a marked upregulation of the maternally expressed ncRNAs of the imprinted *DLK1-DIO3* domain on human chromosome 14. Concordantly, overexpression of IPW lncRNA in iPS cells gave rise to reduced expression of these genes of the *DLK1-DIO3* domain expressed from the maternal chromosome. The precise mechanism through which this lncRNA acts is unclear, but this does not involve altered DNA methylation at the ICR of the *DLK1-DIO3* domain. This discovery, though

still preliminary, emphasizes that imprinted genes are interdependent and co-regulated, at least in part, which agrees with the finding that many mammalian imprinted genes are co-regulated and may form an “imprinted gene network” (Al Adhami et al. 2015; Varrault et al. 2006).

Other imprinted lncRNAs were shown to have functional effects in *trans* as well. One of these is the lncRNA H19, transcribed from the maternal chromosome at the *Igf2-H19* domain on mouse chromosome 7. Using a mouse model of *H19* gene deletion, it was shown that loss of the imprinted *H19* lncRNA engendered an upregulation of at least eight other imprinted genes, residing on six different chromosomes (Gabory et al. 2009). Normal expression levels were restored at these genes following transgene-driven overexpression of *H19* in the knockout mice. The latter finding clearly confirms that the lncRNA itself has a repressive role in *trans*. How, precisely, it affects other imprinted gene loci is still unclear. Earlier studies had found that the *H19* lncRNA recruits the repressive methyl-CpG-binding domain protein MBD1 to DMRs at several imprinted domains (Monnier et al. 2013) and this chromatin modifier could therefore be part of the repressive mechanistic action of H19 lncRNA.

2.5 Long Noncoding RNAs Involved in Imprinted Gene Activation

A new insight is that some mammalian lncRNAs bring about “active” chromatin in *cis* through recruitment of protein complexes that mediate gene transcription and H3K4 methylation (Orom et al. 2010). Well-studied examples of activating lncRNAs include Hottip, which is involved in the developmental activation of the homeotic genes at the *Hoxa* cluster (Wang et al. 2011), and an lncRNA that activates gene expression at the FSHD (facioscapulohumeral dystrophy) disease locus in humans (Cabianca et al. 2012). These conceptually interesting studies have raised the question as to whether some of the imprinted ncRNAs could be involved in transcriptional activation as well.

At the *Dkl1-Dio3* domain, the paternally methylated ICR activates imprinted gene expression on the maternal chromosome in the early embryo. Particularly, targeted deletion of this ICR region on the maternal chromosome gave rise to a complete loss of expression of all the maternally transcribed ncRNAs, including the lncRNA Gtl2/Meg3 (Lin et al. 2003). This enhancer-like action of the ICR on the maternal chromosome was found to be linked to RNA polymerase II recruitment and to bidirectional transcription from the ICR of multiple ncRNAs that are relatively short and non-polyadenylated (Kota et al. 2014). These maternal allele-specific ICR ncRNAs, together with specific histone modifications at the ICR-associated chromatin (Kota et al. 2014), are similar to those reported at other active enhancers (Natoli and Andrau 2012). It is not known how ICR “enhancer RNAs” (or their transcription) activate gene transcription in *cis*. In embryonic stem

and iPS cells, however, the expression of these small RNAs correlates with an early timing of DNA replication in S phase and with a more central subnuclear localization of the locus compared to that of the paternal chromosome (Kota et al. 2014). Stable shRNA-mediated knockdown of the ICR ncRNAs led to a lack of gene activation in *cis*, and this correlated with delayed replication timing on the maternal chromosome (Kota et al. 2014).

A second ICR that activates genes in *cis* is the intragenic ICR controlling the Prader-Willi syndrome (PWS) imprinted domain (also called the *Snrpn* domain) on mouse chromosome 7. This 2-Mb imprinted domain comprises multiple genes that are activated on the paternal chromosome, particularly during neurogenesis (Lee and Bartolomei 2013; Peters 2014). The unmethylated paternal copy of this intragenic ICR controls the gene activation in *cis*, which occurs on one side of the domain at more than 1.5 Mb away from the ICR. Recent research indicates that the enhancer-like function of the ICR is linked to chromosome looping, bringing the paternal copy of the ICR in close proximity to the distant target genes (Rabinovitz et al. 2012). Additionally, the non-methylated paternal copy of the ICR drives the expression of an lncRNA, called *Snurf/Snrpn*. It is unknown whether this long noncoding RNA is functional and required for the long-distance chromatin regulation as well.

2.6 Conclusions and Future Directions

Considerable progress has been made in our understanding of genomic imprinting in mammals. Many novel insights have emerged, particularly into the essential somatic maintenance of the differential DNA methylation at ICRs in the embryo. These have improved our understanding of imprinting disorders in humans (Girardot et al. 2013). In fact, it has been largely through clinical genetic studies that several of the nonhistone proteins involved were discovered and could be tested in animal studies subsequently. Above, I reviewed the importance of chromatin features and regulatory ncRNAs in the tissue specificity of imprinted gene expression. At several domains, repressive histone methylation is mediated by PRC complexes and KMTs, and at some domains, there is involvement of lncRNAs in the recruitment/activity of chromatin regulatory complexes as well. We now understand better how chromatin repression, long-range looping, and regulatory lncRNAs contribute together to imprinted expression. Long-range interactions can now be studied genome wide, using high-throughput 3C and DNA sequencing technologies. Such approaches, combined with efficient technologies to target key elements (e.g., CTCF-binding sites), will undoubtedly provide further insights into the developmental structuration of imprinted domains. Intriguing mechanistic similarities between different domains have emerged as well, particularly as concerns placental imprinting.

A lot is still to be discovered though about factors defining the tissue specificity of imprinting. One might expect transcription factors to play pivotal roles in

guiding chromatin configuration and gene expression. The recent studies on the PWS and *DLK1-DIO3* imprinted domains emphasize that factors that control enhancer activity are likely to be involved as well. Given the growing interest in this fast-moving field, undoubtedly, novel regulatory factors and mechanisms will be discovered during the years to come.

Acknowledgments I thank all members of the *Genomic Imprinting and Development* laboratory for discussion and comments. Our discussed research is grant funded by the National Research Agency (ANR) and the Institut National Contre le Cancer (INCa). Our laboratory is affiliated to the Montpellier Laboratory of Excellence “EPIGENMED,” the EU 7th Framework COST Action “Human Congenital Imprinting Disorders,” and the EU 7th Framework Network of Excellence “EpiGeneSys.”

Conflict of Interest The author declares no conflict of interest.

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Epigenetics - A Different Way of Looking at Genetics

Doerfler, W.; Böhm, P. (Eds.)

2016, XVI, 254 p. 41 illus., 9 illus. in color., Hardcover

ISBN: 978-3-319-27184-2