Genomic imprinting in germ cells: imprints are under control

Philippe Arnaud

Institut de Génétique Moléculaire, CNRS UMR-5535 and Universités de Montpellier, 1919, route de Mende, 34090 Montpellier, France

Correspondence should be addressed to P Arnaud; Email: philippe.arnaud@igmm.cnrs.fr

Abstract

The cis-acting regulatory sequences of imprinted gene loci, called imprinting control regions (ICRs), acquire specific imprint marks in germ cells, including DNA methylation. These epigenetic imprints ensure that imprinted genes are expressed exclusively from either the paternal or the maternal allele in offspring. The last few years have witnessed a rapid increase in studies on how and when ICRs become marked by and subsequently maintain such epigenetic modifications. These novel findings are summarised in this review, which focuses on the germline acquisition of DNA methylation imprints and particularly on the combined role of primary sequence specificity, chromatin configuration, non-histone proteins and transcriptional events.

Introduction

In the mid-1980s, elegant embryological studies in the mouse demonstrated the functional non-equivalence of the maternal and paternal genomes. Specifically, conceptuses derived from zygotes containing either two sets of the maternal chromosomes or two sets of the paternal chromosomes failed to develop beyond mid-gestation (Barton et al. 1984, McGrath & Solter 1984). These findings demonstrated that normal embryonic development requires both a maternal and a paternal genome and suggested the existence of genes whose expression depends on whether they are inherited from the mother or from the father. This specific regulation of gene expression was named ‘genomic imprinting’, and the genes involved was named as ‘imprinted genes’. Since then, more than 100 of imprinted genes have been identified in the mouse, and the imprinting of many of them, though not of all, is conserved in human as well. About half of the imprinted genes are expressed from the maternal allele and the other half from the paternal allele. Imprinted genes are found throughout the genome. Some of them appear to be isolated, but the majority of them reside in domains that cover hundreds to a thousand of kilobases. These imprinted clusters comprise both maternally and paternally expressed genes as well as non-imprinted genes, and usually, they contain at least one non-coding RNA (Fig. 1).

Genomic imprinting is conserved among viviparous mammals, especially eutherians, and, to a lesser extent, in marsupials. Imprinting has not been detected in egg-laying monotremes (Rentree et al. 2009). Up-to-date repertoires of imprinted genes, their diverse functions and their imprinted status in several mammalian species (if known) can be found elsewhere (see ‘Websites of interest’ section). Briefly, many imprinted genes have roles in the regulation of foetal and/or placental growth and development. Others play key roles in neurological pathways and in behaviour (Smith et al. 2006, Wilkinson et al. 2007). As a consequence, perturbations in imprinted gene expression are an important cause of several growth and behavioural syndromes in humans including the Silver–Russell, Beckwith–Wiedemann, Prader–Willi and Angelman syndromes (Hirasawa & Feil 2010).

The allele-specific expression of imprinted genes (and more broadly of imprinted loci) is regulated by epigenetic modifications that differentially mark the parental alleles as either active or repressed at discrete cis-acting elements known as imprinting control regions (ICRs). These regions exhibit patterns of parental allele-specific DNA methylation, which are essential for orchestrating mono-allelic gene expression. The last few years have witnessed a rapid increase in studies on how and when ICRs become marked by epigenetic modifications. These recent findings are described in this review with emphasis on the mechanisms that are involved in the acquisition of imprints in the germlines.

Differentially methylated regions control imprinting

Virtually all examined imprinted loci contain a differentially methylated region (DMR) of up to several kilobases in size that is characterised by DNA methylation marks inherited from the male or the female gamete (the germline DMRs). To date, 21 of such germline DMRs have been identified in the mouse (Fig. 1), and indirect
Reprogramming of imprints between generations

Imprints need to be reset at each new generation. First, the existing imprints at ICRs are erased, and then, a new set of imprints according to the sex of the developing germ cells is acquired. Subsequently, these imprint marks are acquired in the germ cells, DNA methylation imprints are maintained in all the somatic lineages throughout the development (Fig. 2), where the marks are ‘read’ in different ways to ensure appropriate parental allele-specific expression. A detailed description of the reading mechanisms is outside the scope of this review, and can be found in other comprehensive review texts (Ideraabdullah et al. 2008, Bartolomei 2009). Schematically, differential DNA methylation of germline DMRs/ICRs is thought to initiate a sequence of events that ultimately gives rise to coordinated allele-specific expression for clusters of imprinted genes that are up to one megabase in size (Fig. 1). The simplest scenario is when the ICR is also a promoter, thus leading to direct silencing of only the methylated allele. In the case of large imprint domains, associated ‘long distance’ regulatory modules, mainly through chromatin insulators and non-coding RNAs, are also required. Importantly, for many imprinted genes, these ‘reading’ mechanisms and the ensuing imprinted gene expression are limited to specific tissues or developmental stages, despite the constitutive presence of the allelic DNA methylation imprint. Methylation at ICRs can thus be considered as the first, necessary level of control that acts upstream of diverse tissue- and developmental-specific regulatory mechanisms.
maintained from the zygote to all somatic tissues (Fig. 2). Because of the fundamental role of DNA methylation in conferring a functional imprint (Obata & Kono 2002), the ‘imprint life cycle’ can be recapitulated by the methylation dynamics at germline DMRs. Primordial germ cells (PGCs) originate from 7 days post coitum (dpc)-old proximal epiblast and, following migration, colonise the developing gonads at around 10.5 dpc to differentiate into gametes. At 13.5 dpc, female germ cells enter meiosis, whereas male germ cells undergo mitotic arrest. During this developmental window, germ cells of both sexes undergo a major epigenetic reprogramming believed to be necessary to restore totipotency. This reprogramming occurs in two steps. First, a (possibly passive) decrease in DNA methylation, which is associated with reprogramming of histone modifications, initiates in migrating PGCs at around 8 dpc (Seki et al. 2005, Hajkova et al. 2008). After they have reached the gonads (at around 10.5 dpc), PGCs undergo a second (possibly active) genome-wide DNA demethylation process. Importantly, all sequences in the genome do not seem to be affected equally by these two demethylation steps. Particularly, erasure of genomic imprints occurs only during the second (active) step, since ICRs retain their methylation marks up to 11.5 dpc before undergoing rapid demethylation that is complete by 13.5 dpc (Hajkova et al. 2002; Fig. 3). Although the precise mechanism is not fully elucidated, recent studies show that the activation-induced cytidine deaminase (AID) contributes to this second demethylation step (Popp et al. 2010), and that histone replacement might also play an important role in this active process (Hajkova et al. 2008).

New methylation imprints are acquired at later stages of mouse germ cell development, with a different timing in the two sexes (Fig. 3). In the male germline, paternal imprints are acquired in mitotically arrested germ cells. Imprint establishment starts before birth, in pro-spermatogonia (at around 14.5 dpc), is completed in peri-natal pro-spermatogonia, and is maintained through the meiotic and haploid stages (Davis et al. 2000, Kato et al. 2007).

Maternal imprints, in contrast, are acquired after birth only, during oocyte growth (Fig. 3). This happens in cells that have already initiated meiotic recombination (with 4n DNA content), and is dependent on the size of the oocyte (Lucifero et al. 2004, Hiura et al. 2006). Maternal methylation marks are acquired asynchronously at different loci: some germline DMRs, such as Snprn and Peg3, are methylated earlier, while others, like the Mest DMR, become methylated at later stages of oocyte growth. Nonetheless, maternal methylation marks are fully acquired by the metaphase II stage.
Interestingly, the timing of methylation acquisition could be different in the two alleles of DMRs. Davis et al. (2000) reported that in male germ cells, DNA methylation at the H19 DMR is first acquired on the paternally inherited allele (i.e. the previously methylated allele). Similar observations were made in the female germ line for the Snrpn, Mest and Plagl1 DMRs, which first acquire DNA methylation on the maternally-inherited allele in growing oocytes (Lucifero et al. 2004, Hiura et al. 2006). These observations suggest that both in male and in female germ cells, a ‘signal’, possibly involving other epigenetic modifications, could allow parental alleles to remember their origin and influence the process of reacquisition of methylation.

Timing of imprint establishment in humans is less well documented. The H19 ICR (putative) is unmethylated in foetal male germ cells. Methylation starts to be acquired in adult spermatogonia only, before meiotic division (Kerjean et al. 2000). In the female germ line, only a limited number of studies are available. In one detailed analysis, methylation at the KvDMR1 was shown to be acquired progressively during oocyte maturation and to be completed by the metaphase II stage (Khoeiry et al. 2008). Similarly, the SNRPN DMR was found to be fully methylated in mature oocytes (Geuns et al. 2003), suggesting a timing similar to that observed in the mouse. However, in another study, the SNRPN DMR was reported to be unmethylated in mature oocytes, suggesting that this methylation mark is acquired only upon, or after, fertilisation in humans (El-Maarri et al. 2001).

Once acquired in the germline, and following fertilisation, methylation imprints persist throughout the development and adult life. This occurs in the context of the genome-wide reprogramming processes that characterise the pre- and post-implantation stages (Morgan et al. 2005; see below). In conclusion, the developmental life cycle of imprinting appears to be a complex and tightly regulated process that is likely to be mediated by several other factors in addition to the DNA methylation machinery.

Factors that mediate imprint establishment

The methylation machinery involved in imprint acquisition in the female germline is well characterised. A key factor is DNMT3L, a protein that belongs to the DNMT3 de novo methyltransferase family even though it lacks a functional methyltransferase domain. In DNMT3L−/− mice, germline DMRs (as observed for Snrpn, Igf2r and Peg3) failed to be methylated in oocytes (Bourc’his et al. 2001, Lucifero et al. 2007), and consequently, DNMT3L−/− females generate embryos, in which virtually all ‘maternal’ germline DMRs lack methylation, whilst, importantly, methylation of the rest of the genome is apparently unaffected (Fig. 4). Because of the deregulated expression of associated imprinted genes (aberrant bi-allelic expression or complete repression), these conceptuses arrest development before embryonic day 10.5 (Bourc’his et al. 2001, Hata et al. 2002).

Biochemical and genetic studies demonstrated that DNMT3L is an essential factor of maternal imprint establishment through its interaction with de novo methyltransferase DNMT3A (Kaneda et al. 2004, Suetake et al. 2004).

Although there are some discrepancies, studies in the male germline support the idea that the acquisition of
‘paternal’ methylation imprints also relies on DNMT3L (Fig. 3). Specifically, the DNMT3A–DNMT3L complex contributes to methylation acquisition at the H19 and IG-DMR DMRs (Bourc’his & Bestor 2004, Webster et al. 2005, Kato et al. 2007). On the other hand, DNA methylation at the Rasgrf1 DMR is only slightly affected in Dnmt3a−/− or Dnmt3b−/− male germ cells, suggesting that both enzymes are redundantly involved in association with DNMT3L (Kato et al. 2007; Fig. 4). It should be noted, however, that unlike the female germ line, where absence of DNMT3A or DNMT3L leads to complete failure of methylation imprint acquisition, ‘paternal’ germine DMRs are only partially unmethylated in Dnmt3a−/− or Dnmt3l−/− male germ cells. This suggests that DNMT3A and DNMT3B are functionally redundant in the male germ line, and/or that other yet to be characterised factors are involved. In contrast to the female germ line, DNMT3L shows a broader action in the male germ line as it is also responsible for the de novo methylation and transcriptional silencing of dispersed repeated sequences in spermatogonia (Bourc’his & Bestor 2004, Webster et al. 2005, Kato et al. 2007).

In summary, altogether these findings indicate that the DNMT3A/DNMT3L complex is the core of the DNA methylation machinery involved in imprint acquisition in both female and male germ line (Fig. 3). How, precisely, this complex is targeted to ICRs in a germ line-specific manner is not known. Recent studies suggest that several components could contribute to this process, including primary sequence specificity, chromatin configuration, non-histone proteins and transcriptional events.

Imprinting acquisition: sequence specificities

Both in mice and humans, the genomic features of paternal and maternal germ line DMRs are clearly different. Maternal germ line DMRs are enriched in CpG dinucleotides, most of them fulfilling the criteria of a CpG island, and they are always located in a promoter region. By contrast, all the four known paternally methylated germ line DMRs reside in intergenic regions, and are CpG poor. It is somehow intriguing that most of the maternal germ line DMRs are CpG islands as this class of sequences is normally unmethylated in the genome, suggesting a sequence-specific capacity of ‘imprinted’ CpG islands to attract DNA methylation. It was postulated that arrays of tandem repeat motifs (Neumann et al. 1995), which are frequently found in or close to germine DMRs (Neumann et al. 1995, Hutter et al. 2006), could have a role in imprint acquisition. Such repeats, however, have been convincingly shown to be necessary for correct differential methylation only at the Rasgrf1 locus, whereas they are dispensable for imprint acquisition at.

Table: Genomic imprinting in germ cells

<table>
<thead>
<tr>
<th>Name of factors</th>
<th>Type of factor</th>
<th>Germline DMR(s) known to be affected by its deletion</th>
<th>Germline DMR(s) known not to be affected by its deletion</th>
<th>Comments (see text for details)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT3L</td>
<td>Non-catalytic co-factor of Dnmt3a/b</td>
<td>All germline DMRs tested so far (i.e. KvDMRs, Gi601, U2af1-sil, Plag1, Sraip, Gnalpha3, Nap1, 5′pg; 5′pg, IGF2r, Peg10, Meta2, H19, Rasgrf1, IG-DMR)</td>
<td>Rassf1?</td>
<td>- Depletion effect is less severe in male than in female germine</td>
<td>Bourc’his et al. (2001)</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>DNA methyltransferase with de novo activity</td>
<td>All germline DMRs tested so far (i.e. Sraip, Peg1; Peg3; IGF2r, H19; IG-DMR)</td>
<td>Rasgrf1?</td>
<td>- DNMT3A and DNMT3L are thought to act redundantly on Rasgrf1 DMR</td>
<td>Webster et al. (2005)</td>
</tr>
<tr>
<td>KDM1B</td>
<td>H3K4me2-histone demethylase</td>
<td>Meas; GobH10; Plag1, Impact</td>
<td>Rasgrf1?</td>
<td>- DNMT3A and DNMT3L are thought to act redundantly on Rasgrf1 DMR</td>
<td>Hata et al. (2002)</td>
</tr>
<tr>
<td>ZFP57 (maternal)</td>
<td>Transcription factor of the KRAF zinc-finger protein family</td>
<td>Sraip</td>
<td>Rasgrf1?</td>
<td>- DNMT3A and DNMT3L are thought to act redundantly on Rasgrf1 DMR</td>
<td>Kaneda et al. (2004) and Kato et al. (2007)</td>
</tr>
<tr>
<td>NLRP3 (human)</td>
<td>Members of the CATERPILLER protein family involved in inflammation and apoptosis</td>
<td>PEG3, SRNP1, KdMDR1, Cnas1a</td>
<td>PEG3</td>
<td>- DNMT3A and DNMT3L are thought to act redundantly on Rasgrf1 DMR</td>
<td>Henckel et al. (2009)</td>
</tr>
<tr>
<td>NLRP2 (human)</td>
<td>KdMDR1 and SRNP1 and Plag1DMRs</td>
<td>SRNP1 and Plag1DMRs</td>
<td>SRNP1 and Plag1DMRs</td>
<td>- DNMT3A and DNMT3L are thought to act redundantly on Rasgrf1 DMR</td>
<td>Meyer et al. (2009)</td>
</tr>
<tr>
<td>Transcription-through events</td>
<td>Germine DMRs at Gnas domain (GnasX1 and 1A DMRs)</td>
<td>Also detected at: IGF2r, GobH10, Plag1, Impact and Kvdmr1</td>
<td>Also detected at: IGF2r, GobH10, Plag1, Impact and Kvdmr1</td>
<td>- DNMT3A and DNMT3L are thought to act redundantly on Rasgrf1 DMR</td>
<td>Chotalia et al. (2009)</td>
</tr>
</tbody>
</table>

Figure 4: Factors known to be involved in germline establishment of imprint in mouse and human (when indicated). Maternally methylated germline DMRs are in red, and paternally methylated DMRs are in blue.
the H19 and the KvDMR1 DMRs (Reed et al. 2001, Yoon et al. 2002, Mancini-Dinardo et al. 2006). The CpG spacing at the targeted sequences might be more relevant for imprint acquisition. Structural analyses showed that DNMT3A and DNMT3L form a tetrameric complex that preferentially methylates pairs of CpGs that are 8–10 bp apart (Jia et al. 2007). Such CpG spacing has been observed at all maternally methylated germline DMRs but not at the paternally methylated germline DMRs so far. The distribution of CpGs could help explaining why the absence of DNMT3L, though affecting both germ lines, has a more drastic effect on DMRs that are methylated in oocytes than on those methylated in sperms (see above). Nevertheless, CpG spacing is probably not specific to germline DMRs as it is found in many other CpG islands in the human genome as well (Ferguson-Smith & Greally 2007). This finding suggests that CpG spacing, if involved in imprint acquisition, does not act on its own, but synergistically with other components.

**Imprint acquisition: a chromatin connection**

Concerning other possible components involved in imprint acquisition, it is interesting to note that in addition to DNA methylation, germline DMRs are also marked by allelic histone modifications in somatic cells. Several studies, initially based on locus-specific analysis of histone modifications and further supported by genome-wide analyses, revealed the existence of a germline DMR-specific chromatin signature. Specifically, at both maternal and paternal germline DMRs, the DNA unmethylated allele is associated with H3/H4 acetylation and dimethylation of lysine 4 of histone H3 (H3K4me2), hallmarks of permissive chromatin. By contrast, the DNA methylated allele is consistently associated with histone marks that are characteristic of repressive chromatin: tri-methylation on lysine 9 of histone H3 (H3K9me3), trimethylation on lysine 20 of histone H4 (H4K20me3) and symmetrical dimethylation on arginine 3 of histones H4/H2A (H4/H2AR3me2s; Henckel et al. 2009). Based on observations in other organisms, particularly in the fungus Neurospora crassa, where it has been clearly established that H3K9me3 is a prerequisite for DNA methylation (Tamaru et al. 2003), it is tempting to speculate that this (or part of) specific chromatin signature could be involved in recruiting the DNMT3A/DNMT3L complex. However, due to technical limitations, it is currently unknown whether these specific histone marks are also present in the germ cells, particularly in growing oocytes. Of concern, nonetheless, is the recent observation that mouse embryos derived from Dnmt3l−/− oocytes (i.e. that failed to acquire their DNA methylation imprint) are devoid of repressive histone marks at the maternal germline DMRs (Henckel et al. 2009). This finding suggests an upstream role for DNA methylation and challenges the classical model, in which repressive histone marks are a prerequisite for DNA methylation imprints. Consistently, the emerging, alternative model does not implicate repressive histone marks in the maternal imprint acquisition, but rather, involves the specific erasure of a permissive histone mark. In agreement with such a scenario, biochemical approaches have shown that DNMT3L interacts with histone H3 only when it is unmethylated on lysine 4, suggesting that H3K4 methylation (permissive mark) prevents the recruitment of DNMT3A/DNMT3L to germline DMRs (Ooi et al. 2007). The proof that H3K4 methylation needs to be removed to allow DNA methylation to occur is brought about by a recent functional study on the histone demethylase KDM1B (Ciccone et al. 2009). This enzyme is an H3K4 demethylase with specificity towards H3K4me2. Its targeted disruption has no effect on somatic development; however, embryos derived from KDM1B−/− oocytes show gross developmental defects, and do not survive beyond 10.5 dpc. This phenotype is reminiscent of the developmental defects observed in the progeny of Dnmt3l−/− females. Consistently, the authors demonstrate that in the absence of KDM1B, a subset of germline DMRs fails to acquire their methylation imprint in oocytes, leading to aberrant expression of the associated genes in the derived embryos. This defect is probably specific to these regions as methylation at several classes of repetitive elements was unaffected. KDM1B appears thus to be a new key player specifically involved in imprint acquisition (Ciccone et al. 2009; Fig. 3). However, unlike DNMT3L, its action is limited to a subset of maternal germline DMRs (Fig. 4).

A similar mechanism could also be present in the paternal germline, but it would be independent of KDM1B, which is expressed almost exclusively in growing oocytes. Alternatively, indirect evidence suggests an implication of repressive marks in paternal imprint acquisition. Experiments conducted in Xenopus laevis oocytes demonstrated that to fully acquire its methylation imprint, the mouse H19 DMR required, in addition to plasmids encoding the methylation machinery (DNMT3A/DNMT3B/DNMT3L) and the cofactor CTCF-like (CTCF; see below) also the arginine methyltransferase PRMT7, suggesting a role for histone arginine methylation in this process (Jelinic et al. 2006). Whether this mark is involved in paternal imprint acquisition at the endogenous locus remains however to be established. On the other hand, H3K9me3 and H4K20me3, two repressive marks constitutively associated with the methylated allele in somatic cells, can probably be discarded for a role in imprint acquisition. Indeed, at a late spermatogenesis stage (i.e. following methylation imprint acquisition), the H19 and IG-DMR DMRs do not show enrichment for these marks, suggesting that they are acquired after DNA methylation (Delaval et al. 2007).
**Imprint acquisition: a role for non-histone proteins**

In addition to the components of the methylation machinery, several recent studies underline the role of other factors in imprint acquisition. These include the KRAB zinc finger protein ZFP57. Following both zygotic and oocyte-specific depletion of Zfp57 in the mouse, Li et al. (2008) demonstrated that besides maintaining DNA methylation at several germline DMRs (see below, Fig. 5), ZFP57 is also required for acquisition of methylation in oocytes at the Snrpn ICR (Fig. 4). Two members of the nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin domain (NLRP) family also are involved in the acquisition of methylation imprints in humans. Specifically, in a recent study conducted on a familial case of the growth disorder Beckwith–Wiedemann, a mutation in the NLRP7/NLRP gene was found to be associated with absence of methylation at the KVDMR1 and PEG1 DMRs (Meyer et al. 2009). Furthermore, a subset of familial hydatidiform moles (FHM) that are abnormal forms of pregnancy, which are thought to result from a genetic defect which prevents the establishment of maternal germline imprints (Judson et al. 2002, Djuric et al. 2006, Murdoch et al. 2006, Kou et al. 2008), has been associated with a mutation in NALP7/NLRP7 (Murdoch et al. 2006, Kou et al. 2008). How mutation in NLRP7 genes leads to methylation imprint defects is unclear. Of interest is the observation that NLRP7 mutations can be associated with partial disease penetrance; some women that carried an NLRP7 mutation nevertheless had a normal pregnancy (Moglaby et al. 1999, Murdoch et al. 2006), and some cases of FHM present abnormal gains of methylation at the H19 DMR (El-MMaarri et al. 2003). Combined, these observations indicate that mutations in NLRP7 lead to a complex and heterogeneous pattern of abnormal methylation imprints, and suggest a role in both germline acquisition and post-zygotic maintenance.

Finally, the CTCFL protein, also known as BORIS, is a factor, which might be involved in imprint acquisition at the paternally methylated H19 DMR. CTCFL is a testis-specific parologue of CTCF, an insulator protein that binds to the unmethylated H19 DMR. Immunoprecipitations conducted on chromatin purified from embryonic day 15.5 testes have suggested that CTCFL is physically bound to the H19 ICR in male germ cells. In addition, CTCFL interacts with PRMT7, and the experiments conducted in Xenopus oocytes further support the role of these two factors in establishing the methylation imprint at the H19 DMR (Jelinic et al. 2006). However, it remains to be formally demonstrated that CTCFL and PRMT7 are indeed involved in imprint acquisition in the mammalian male germ line, particularly in view of the fact that functional CTCF-binding sites are dispensable for methylation imprint acquisition at the H19 DMR during spermatogenesis (Engel et al. 2006).

**Imprint acquisition: a role for transcripational events**

The role of small RNAs in triggering de novo DNA methylation is well documented, especially in the plant kingdom (Matzke et al. 2009). In mammals, de novo DNA methylation of repetitive elements (IAP and Line-1) during spermatogenesis appears to be

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**Table:**

<table>
<thead>
<tr>
<th>Factors known to be involved in methylation maintenance</th>
<th>Type of factor</th>
<th>Germline DMR(s) known to be affected by its deletion</th>
<th>Germline DMR(s) known not to be affected by its deletion</th>
<th>Comments (see text for details)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZFP57 (maternal+zygotic)</td>
<td>Transcription factor from KRAB Zinc-finger protein family</td>
<td>In mouse: Snrpn, Peg1, Peg3, Nnat; IG-DMR</td>
<td>In human: PLAGL1, GRB10 and PEG3</td>
<td>In mouse: IgG2r (partial effect?)</td>
<td>Protect the methylated allele against the pre-implantation DNA demethylation wave</td>
</tr>
<tr>
<td>PGC7/Stella</td>
<td>Maternal factor essential for early development</td>
<td>Peg1; Peg3; Peg10, H19; Rasgrf-1</td>
<td>Snrpn, Nnat; IG-DMR</td>
<td></td>
<td>Nakamura et al. (2007)</td>
</tr>
<tr>
<td>MBD3</td>
<td>Methyl CpG binding protein 3</td>
<td>H19</td>
<td>Snrpn, indirect evidences for Peg3 and IG-DMR</td>
<td>Methylation is reduced but not abolished</td>
<td>Reese et al. (2007)</td>
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<tr>
<td>RBBP1 and RBBP1-like 1</td>
<td>RB binding proteins</td>
<td></td>
<td></td>
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<td>Wu et al. (2006)</td>
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<td>Linker-histone H1</td>
<td></td>
<td>H19 and IG-DMR</td>
<td></td>
<td>Methyltion is reduced but not abolished</td>
<td>Fan et al. (2005)</td>
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<td>Factors known to be involved in protecting against methylation</td>
<td>Type of factor</td>
<td>Germline DMR(s) known to be affected by its deletion</td>
<td>Germline DMR(s) known to be affected by its deletion</td>
<td>Comments</td>
<td>References</td>
</tr>
<tr>
<td>CTCF</td>
<td>Insulator protein</td>
<td>H19</td>
<td></td>
<td>Protect unmethylated allele during post-implantation de novo methylation wave</td>
<td>Engel et al. (2003) and Schoenherr et al. (2003)</td>
</tr>
</tbody>
</table>

**Figure 5** Factors known to be involved in imprint maintenance in mouse and human (when indicated). Maternally methylated germline DMRs are in red, and paternally methylated germline DMRs are in blue.
regulated by MILI and MIWI2, two Piwi-interacting small RNA (piRNA)-binding proteins (Kuramochi-Miyagawa et al. 2008). Although these pathways might provide an attractive mechanism for imprint establishment, their involvement in this process has not been demonstrated so far (see Kačem & Feil 2009 and ref inside). Similarly, it is well documented that non-coding RNAs are involved in imprinting (Ideraabullah et al. 2008). However, the described imprinted non-coding transcripts are controlled by the DNA methylation imprints, and their function so far seems limited to ‘reading’ the imprints rather than in their establishment.

Evidence for a transcription-based event in imprint acquisition comes from the Gnas locus. This complex locus comprises a series of imprinted overlapping coding and non-coding transcripts, and is characterised by the presence of two maternally methylated germline DMRs (Fig. 1). An original study (Chotalia et al. 2009) demonstrated that truncation of the Nespt transcript, which initiates furthest upstream in the locus and covers the entire domain, disrupts the acquisition of methylation at the two germline DMRs in oocytes. This observation is somehow reminiscent of an earlier study in humans, in which absence of methylation at the GNASXL and 1A DMRs was associated with deletion of the NESP promoter region (Bastep et al. 2005). Combined, these observations suggest that transcription across the germline DMRs of the Gnas locus is required for the establishment of their DNA methylation imprint in oocytes. It is postulated that transcription could serve to ‘open’ the chromatin and facilitate the recruitment of the DNA methylation machinery. The observation that the five other maternal germline DMRs tested are also traversed by protein-coding transcripts (some of which are oocyte specific) in growing oocytes suggests that this newly discovered mechanism might occur at other imprinted domains as well (Chotalia et al. 2009; Fig. 4). Conversely, it is not known whether such a mechanism might also be relevant for the establishment of intergenic paternally methylated germline DMRs.

**Imprinting in the absence of germline DNA methylation acquisition**

The canonical starting point of the imprint cycle is the acquisition of DNA methylation in the germline (Figs 2 and 3). However, several studies suggest that this key step can be by-passed, and that methylation imprints can be acquired later in development. For instance, when the H19 DMR is inserted at ectopic positions in the genome, paternal-specific DNA methylation at this DMR can be established partially, or even completely, after fertilisation, during early development (Park et al. 2004, Tanimoto et al. 2005). Furthermore, methylation imprints at some ICRs can be present in a stochastic manner in some embryos derived from Dnmt3l−/− deficient oocytes (Arnaud et al. 2006). Of particular interest are Snrpn and Peg3 ICRs, which were found to be fully maternally methylated in respectively 30 and 14% of the progeny of Dnmt3l−/− females (Arnaud et al. 2006, Henckel et al. 2009). Since bisulphite analyses in Dnmt3l−/− oocytes failed to detect methylation at these loci (Bourc’his et al. 2001, Lucifero et al. 2007), the methylation imprints observed in the progeny (in both 9.5 dpc embryos and trophoblast cells) must have been acquired at the imprint stage (Henckel et al. 2009). Similarly, failure to establish the Snrpn ICR methylation imprint in Zfp57−/− eggs could be partially rescued by the paternal derived ZFP57 at post-implantation stages (Li et al. 2008). These observations, especially at the Snrpn ICR, are consistent with studies that report that methylation at the SNRPN ICR in humans might occur after fertilisation (El-Maarri et al. 2001, Kaufman et al. 2009; note, however, that Geuns et al. (2003) found that the human SNRPN ICR is methylated already in oocytes).

Combined, these analyses suggest that (some) ICRs carry a germline-derived (DNA methylation independent) signature that can mediate DNA methylation acquisition on the right parental allele at implantation during the genome-wide de novo methylation. The nature of such signature and whether the ‘embryonic’ establishment recapitulates the mechanism that normally occurs in the germline remains to be discovered. However, it is tempting to speculate that a germline-derived chromatin signature and/or transcription through ICR at implantation will be recognised by the somatic DNMT3A/DNMT3L complex and by relevant other non-histone proteins. The post-fertilisation establishment of DNA methylation imprints could constitute a safeguard system to sense and correct imprint methylation defects that could otherwise lead to serious developmental defects.

**Factors that mediate the maintenance of imprints**

Following their establishment, methylation imprints have to be maintained and faithfully transmitted to all somatic lineages. Remarkably, this occurs despite genome-wide reprogramming events. Indeed, the first issue is to understand how the paternal imprints resist the active demethylation of the paternal genome that occurs after fertilisation (Morgan et al. 2005). One exciting hypothesis comes from the observation that in the human sperm, ICRs and some developmentally regulated genes are apparently not subject to histone-to-protamine exchange (Hammoud et al. 2009). This could be consistent with the involvement of a specific chromatin structure in the transmission of the paternal imprint from spermatozoa to the zygote. Following fertilisation and up to implantation, the methylated alleles of all germline DMRs are protected from the global wave of demethylation. Continuous expression of maternal and then zygotic Dnmt1 in pre-implantation embryos are required for this process (Biniszkiewicz et al. 2002, Hirasawa et al. 2008).
Besides, the methylation machinery, other protein factors are involved in maintaining parental-specific imprints. One of these is the KRAB zinc finger protein ZFP57. In addition to being involved in the acquisition of the methylation imprint at the Snrpn ICR, ZFP57 is also required to maintain DNA methylation at several paternal and maternal imprinted regions (Li et al. 2008; Fig. 5). The role of ZFP57 in maintaining methylation imprints is conserved between mice and humans as suggested by a study in patients with transient neonatal diabetes mellitus (TNDM; Mackay et al. 2008). In these patients, mutations in ZFP57 were associated with hypomethylation at the (putative) ICR of PLAGL1, a defect causally involved in TNDM. Interestingly, these patients often present hypomethylation at other ICRs as well (Mackay et al. 2008). How ZFP57 mediates its action is unknown. However, this class of transcription factors is thought to act as transcriptional repressors by recruiting the KAP-1/TIF1B repressive complex (Abrink et al. 2001). This complex includes histone methyltransferases and deacetylases potentially involved in the maintenance of DNA methylation imprints. Another relevant protein is PGC7/Stella, a maternal factor that is required for embryonic development and partially protects against loss of DNA methylation at specific paternal and maternal ICRs (Fig. 5; Nakamura et al. 2007). Interestingly, ZFP57 and Stella have only partially overlapping effects (Fig. 5). The methyl CpG-binding domain protein 3 (MBD3) was also found to be involved in imprint maintenance, though its role appears limited to the paternally methylated H19 ICR (Reese et al. 2007). This was unexpected, since MBD3 itself does not bind directly to methylcytosines indicating that maintenance could rely on MBD3-containing protein complexes such as NuRD.

The last step of the early development reprogramming (after the wave of demethylation) is characterised by a wave of genome-wide de novo methylation that occurs soon after implantation. The specific association of the unmethylated alleles of germline DMRs with H3K4me2, a mark linked with chromatin that is permissive for transcription, probably contributes to protect DMRs from aberrant acquisition of methylation (Ooi et al. 2007, Ciccone et al. 2009). Besides chromatin structure, the CTCF zinc finger domain protein is also implicated in protection against DNA methylation. Indeed, in absence of CTCF binding, the mouse H19 ICR is susceptible to acquire methylation at the normally unmethylated parental allele during post-implantation de novo methylation (Schoenherr et al. 2003, Engel et al. 2006; Fig. 5).

The interplay between histone and DNA methylation is thought to be important for the somatic maintenance of imprints. The recent observation that absence of methylation imprints acquisition drastically affects the chromatin signature at ICRs is consistent with the existence of a mechanistic link between both types of methylation marks (Henckel et al. 2009). However, functional evidence for a role of repressive histone marks in imprint maintenance has not been documented. Of relevance could be the observation that DNA methylation is reduced at several ICRs regions in ES cells that lack the G9a H3K9me2 HMT (Dong et al. 2008, Tachibana et al. 2008). However, this is more likely due to the absence of G9a itself rather than to the loss of H3K9me2 (Dong et al. 2008, Tachibana et al. 2008). Furthermore, this observation was not confirmed in 9.5 dpc G9a+/− embryos (Tachibana et al. 2008). Absence of H4K20me3 (another chromatin repressive mark) also failed to affect methylation imprint maintenance in vivo (Wu et al. 2006, Pannetier et al. 2008), thus questioning the role of this histone modification at ICRs. Indirect evidence indicates a role for chromatin in imprint maintenance. In ES cells depleted of the three subtypes of the linker histone H1, hypomethylation and aberrant expression of the associated genes were observed at the H19 and IG-DMR ICRs, while most of the genome remained unaffected (Fan et al. 2005). Similarly, RBBP1 and RBBP1-like1, two RB-binding proteins, are required for maintenance of both DNA and histone (H4K20me3 and H3K9me3) methylation marks, at the mouse Snrpn ICR (Wu et al. 2006).

Outlooks

In recent years, major advances have been made in our understanding of the mechanisms that govern the establishment of genomic imprints. The emerging picture suggests that CpG spacing, removal of H3K4me and transcriptional read-through are likely to act in a concerted and not exclusive manner to recruit the DNMT3A/DNMT3L complex on ICRs in growing oocytes (Fig. 3). With the notable exception of CpG periodicity, these factors could also play a role in paternal imprint acquisition. Furthermore, recent studies emphasise that imprint acquisition relies on components that could differ not only between the two germlines, but also between ICRs in the same germline. This could probably account for the temporally asynchronous acquisition of imprints at different DMRs in the same germline. It is tempting to speculate that besides the ‘universal’ imprinting regulator DNMT3L, imprint acquisition relies on a common ‘core’ mechanism that is itself controlled by and/or associated with factors acting in an ICR(s)-specific manner. For instance, structural studies on the DNMT3A/DNMT3L complex (Ooi et al. 2007) and the observation that the presence of the permissive mark H3K4me2 is inversely correlated with DNA methylation in mammalian cells supports the idea that erasure of H3K4me could be generally used for imprint acquisition. Nevertheless, the specificity of KDM1B for only a few of the maternal DMRs (Ciccone et al. 2009) suggests that other H3K4 demethylases could be involved at the other ICRs. Similarly, if transcription through ICRs turns out to be generally used in imprint
acquisition, one may expect that the control of such transcripts might rely on a variety of transcriptional factors. Further understanding of the mechanisms that regulate imprint acquisition necessarily requires defining the temporal relationship between transcriptional events, H3K4me erasure and de novo methylation. This will be obviously facilitated by the recent development of sensitive ‘scaled-down’ techniques dedicated to study epigenetic modifications in limited numbers of cells, including at the genome-wide level (Dahl & Collas 2009, Popp et al. 2010). Such approaches will also help to determine whether, besides the absence of H3K4me, other chromatin signatures are involved in this process. Another clue about a putative chromatin structure could arise through biochemical analysis of KDM1B. Several studies indeed showed that histone demethylases can be associated with specific HMTs in the same complex (Agger et al. 2008). For example, UTX, a demethylase for the repressive mark H3K27me3, is associated with MLL2, the H3K4-specific HMT. This supports a dynamic model, in which the removal of a specific histone tag is associated with the addition of another histone methylation mark. Identification of the HMT that associates specifically with KDM1B should help to unravel the chromatin structure that preclude, and eventually control, methylation imprint acquisition.

Further characterisation of HMTs involved in ICR histone modification in somatic cells is also of importance. Except for SUV4-20H, known to regulate H4K20me3 at ICRs (Pannetier et al. 2008), the HMTs involved in the deposition of other repressive marks at ICRs remain to be determined. Their identification would permit to properly establish the nature of the relationship between histone and DNA methylation at ICRs. The observation that DNA methylation imprints are faithfully maintained in MEF cells deficient for SUV4-20H (Pannetier et al. 2008) suggests a redundant role for the panel of histone marks detected at the methylated allele of ICRs. It would thus be important to test, individually and concomitantly, the impact of HMT depletion in germline acquisition and somatic maintenance of DNA methylation imprints.

Mechanisms that protect ICRs against DNA methylation in the opposite germline have also to be taken into consideration. For instance, high level of H3K4me is detected at maternal ICRs in early spermatogonia cells (Delaval et al. 2007), consistent with the idea that this mark could protect against DNA methylation (Ooi et al. 2007). Since all the described maternal germline DMRs coexist with promoters, it would be interesting to investigate whether this feature is associated (or even controlled) by RNA Pol II binding. Interestingly, a recent study on cancer cells suggests that Pol II binding, regardless of the transcriptional activity, is sufficient to protect promoter CpG islands from methylation (Takeshima et al. 2009).

The insights provided by these new studies, combined with the development of sensitive techniques to analyse germ cells, pave the way for an exciting time during which the mechanisms leading to imprint establishment might finally be unravelled.

Websites of interest

Declaration of interest
The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the work reported.

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