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Genomic imprinting in mammals: its life cycle, molecular mechanisms and reprogramming

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Abstract

Genomic imprinting, an epigenetic gene-marking phenomenon that occurs in the germ-line, leads to parental-origin-specific expression of a small subset of genes in mammals. Imprinting has a great impact on normal mammalian development, fetal growth, metabolism and adult behavior. The epigenetic imprints regarding the parental origin are established during male and female gametogenesis, passed to the zygote through fertilization, maintained throughout development and adult life, and erased in primordial germ cells before the new imprints are set. In this review, we focus on the recent discoveries on the mechanisms involved in reprogramming and maintenance of the imprints. We also discuss the epigenetic changes that occur at imprinted loci in induced pluripotent stem cells.

Introduction

In diploid organisms, the maternal and the paternal alleles of most autosomal genes are expressed at similar levels and thus contribute equally to the phenotype. However, in eutherian mammals (such as humans and mice) and marsupials, the parental alleles are not always functionally equivalent. This was first discovered in early 1980s by embryological studies in mice: nuclear transfer experiments using pronuclear stage embryos showed that reconstituted embryos with two maternal genomes and no paternal complement and those with two paternal genomes and no maternal complement never survive beyond mid-gestation. This suggested that the parental genomes are functionally non-equivalent and marked or imprinted differently during male and female gametogenesis [1, 2]. Almost at the same time, genetic experiments using chromosome translocations in mice showed that specific chromosomal segments, but not the entire genome, function differently depending on the parental origin [3]. Then, mouse *Igf2r* was identified as the first imprinted gene in 1991: it was expressed only from the maternal allele [4]. To date, more than 100 imprinted genes have been identified in mice (http://www.mousebook.org/catalog.php?catalog=imprinting), and many of them are also imprinted in humans [5]. All imprinted genes show either maternal-specific or paternal-specific mono-allelic expression, and their proper expression is essential for normal development, fetal growth, nutrient metablism and adult behavior. In humans, genetic and epigenetic disturbances in expression of the imprinted genes can cause well-known malformation disorders, such as Prader-Willi syndrome, Angelman syndrome, Bechwith-Wiedemann syndrome and Silver-Russell syndrome [5-7].

Most of the imprinted genes are found in clusters in the genome, corresponding to the specific chromosomal segments identified by the above genetic studies. Such imprinted clusters often span hundreds to thousands of kilobases. A given imprinted cluster can comprise both paternally and maternally expressed imprinted genes, some of which are non-coding RNAs, and also non-imprinted genes [8-10]. The clusters also contain CpG-rich regions that are DNA methylated only on one of the two parental chromosomes (differentially methylated regions or DMRs). At some DMRs, differential DNA methylation is also observed between sperm and oocytes and therefore gametic in origin. These DMRs are called germ-line or gametic DMRs. In some cases, there is evidence that the germ-line DMR functions as an imprinting control region (ICR), which controls the mono-allelic expression of the imprinted genes and the methylation status of the other DMRs within the cluster [11]. Most of the germ-line DMRs are methylated in the female germ-line and only four DMRs (*H19*, *Dlk1-Gtl2*, *Rasgrf1* and *Zdbf2*) are known to be methylated in the male germ-line [12, 13]. Importantly, mutations in the maintenance DNA methyltranferase DNMT1 disrupt the parental-origin-specific expression patterns of the imprinted genes in mouse embryos [14]. In addition to DNA methylation, other epigenetic modifications and factors, such as histone modifications, insulator proteins (such as CTCF) and long non-coding RNAs, are also involved in imprinting.

The epigenetic modifications including DNA methylation at the germ-line DMRs undergo dynamic reprogramming during germ-cell development but, on the other hand, they are maintained and faithfully propagated throughout embryonic development [11, 15, 16]. The whole process is complex and regulated tightly. Here, we review the recent discoveries on the mechanisms involved in the establishment, maintenance and erasure of the epigenetic imprints.

We also discuss the epigenetic changes observed at imprinted gene clusters in induced pluripotential stem (iPS) cells.

Life cycle of the genomic imprints

The life cycle of the genomic imprints in mammals is schematically shown in Figure 1. The cycle consists of three major steps: establishment, maintenance and erasure, all of which are important for this biological phenomenon. The establishment of the epigenetic imprints occurs in male and female germ cells. In the male germ-line, *de novo* DNA methylation of the four paternally methylated germ-line DMRs occurs progressively in mitotically arrested (G1/G0) prospermatogonia (or gonocytes) after embryonic day 14.5 (E14.5). Then, the paternal methylation imprints become fully established in prospermatogonia by the neonatal stage [17-21]. In the female germ-line, *de novo* DNA methylation initiates asynchronously at different germ-line DMRs during the oocyte growth phase [22, 23]. Growing oocytes are at the diplotene/dictyate stage of meiotic prophase I, and the maternal methylation imprints become fully established by the fully-grown oocyte stage [22, 23]. The establishment of the maternal methylation imprints is correlated with the establishment of the functional imprints, which was shown by the developmental potential of nuclear transferred bi-maternal embryos [24].

The paternal and maternal epigenetic imprints established in the germ-line are transmitted to the zygote through fertilization and maintained faithfully throughout development and adult life. Notably, the methylation imprints at the germ-line DMRs escape from the global epigenetic reprogramming that occurs in pre-implantation embryos [11, 16, 25]. The reprogramming at this stage includes the replacement of protamines by histones in the paternal

genome, active demethylation of the paternal genome [26], and subsequent passive demethylation of both parental genomes [27, 28]. After implantation, the differential methylation at the germ-line DMRs has to survive another global epigenetic change, i.e. *de novo* DNA methylation. While many genes including the pluripotential genes and germ-cell-specific genes become highly methylated in early post-implantation embryos, the unmethylated allele of the DMR has to be protected from this strong wave of *de novo* DNA methylation. In fact, the imprint maintenance is critical for the parental-origin-specific mono-allelic expression of the imprinted genes throughout development.

The last step of genomic imprinting is the erasure of the epigenetic imprints in primordial germ cells (PGCs): this ensures the sex-dependent imprint establishment in later stages of germ cell development as described above. PGCs are specified from the epiblast cells of early post-implantation embryos. Then, PGCs proliferate actively and migrate to the genital ridge, the precursor of the gonads, between E7.25 to E10.5. In this period, the genome of the PGCs undergoes epigenetic reprogramming to restore pluripotency [25, 29, 30], but they appear to retain the functional imprints at most DMRs [31]. Between E10.5 and E12.5, the parental-origin-specific DNA methylation is erased asynchronously at different germ-line DMRs, and the imprinted genes become biallelically expressed or silenced [32, 33]. Consistent with this, the male and female embryonic germ cells derived at this stage have lost the parental-origin-specific DNA methylation at most DMRs [34].

Below we discuss the molecular mechanisms and factors involved in each step of the imprint life cycle. These factors are summarized in Table 1, together with their biochemical

function and target imprinted genes.

Mechanism of imprint establishment in male and female germ cells

Although circumstantial evidence showed that the gamete-specific differential DNA methylation at the germ-line DMRs is the functional imprints, direct evidence for this was lacking for a long time. The identification of the *de novo* DNA methyltransferase family genes dramatically changed this situation. Mammals have two active *de novo* DNA methyltransferases DNMT3A and DNMT3B [35], and a related protein DNMT3L [36, 37]. DNMT3L has no methyltransferase activity, but is highly expressed in germ cells and can form a complex with DNMT3A and DNMT3B. When the genes coding for these proteins were knocked out in the germ-line of mice, it was found that DNMT3A and DNMT3L are required for the establishment of the maternal imprints in growing oocytes [36-39]. Here, embryos derived from the mutant oocytes displayed loss of DNA methylation at the maternal alleles of the DMRs that are normally maternally methylated, and biallelic expression or silencing of the imprinted genes associated with these DMRs [36-39]. It was later confirmed that the mutant oocytes indeed lack DNA methylation at these germ-line DMRs [39]. It was also established that DNMT3B is dispensable for the establishment of the maternal imprints [39].

In the male germ-line, DNMT3A and DNMT3L again play a central role in *de novo* DNA methylation of the germ-line DMRs. In the *Dnmt3a* mutant prospermatogonia, all four paternally methylated germ-line DMRs showed reduced DNA methylation [20, 21, 38]. DNMT3L was also required for the *de novo* DNA methylation of all DMRs examined [20, 38, 40]. By contrast, in the *Dnmt3b* mutants, only the *Rasgrf1* DMR was affected [20, 38].

However, both *Dnmt3a* mutants and *Dnmt3L* mutants displayed meiotic arrest and azoospermia, it was not possible to assess the effect of the loss of DNA methylation at the DMRs on parental-origin-specific mono-allelic expression of the imprited genes in the embryo.

The discovery that the DNMT3A/DNMT3L complex establishes the methylation patterns at the DMRs in both male and female germ-lines raised a question of how this complex finds its sex-specific targets. Although the exact mechanism is still unknown, some interesting findings have been reported. First, based on the structural analysis of the DNMT3A/DNMT3L complex, it has been proposed that DNA regions with an 8-10-nucleotide CpG interval are the preferred substrate of the DNMT3A/DNMT3L complex [41-43]. However, this sequence feature is found not only in the germ-line DMRs but also in many other CpG islands [43]. Second, unmethylated H3K4 has been proposed to serve as the chromatin signature for the recognition by DNMT3L [41, 44]. Indeed, a lysine H3K4 demethylase KDM1B has been shown to be required for the establishment of the maternal imprints at some DMRs (Peg1 (also called *Mest*), *Grb10*, *Zac1* (also called *Plagl1*) and *Impact*) [45]. However, this protein was dispensable for *de novo* DNA methylation of other DMRs examined (*Kcnq1ot1* (also called *Lit1*), *Igf2r* and *Snrpn*). Third, a KRAB zinc finger protein ZFP57 has been shown to be required for the establishment of the DNA methylation imprint at the *Snrpn* DMR in oocytes [46]. However, this protein was dispensable for DNA methylation of other DMRs in oocytes and, furthermore, the functional imprint of the *Snrpn* DMR was preserved or restored after fertilization. Forth, a truncation of the *Nesp* transcripts at the *Gnas* locus in oocytes resulted in a loss of DNA methylation of the germ-line DMR, indicating transcription through the DMR may be necessary to create or maintain an open chromatin environment that allows the

DNMT3A/DNMT3L complex to access to its targets [47]. As the authors found such transcripts in other maternally methylated germ-line DMRs as well, they propose that this may be a common event for the establishment of the maternal methylation imprints in oocytes. Altogether, the mechanism underlying the recruitment of the DNMT3A/DNMT3L complex to specific targets seems complex, and the specificity may be determined by the combination of common factors and locus-specific factors.

Factors involved in imprint maintenance

Once established, the epigenetic imprints must be faithfully inherited to the zygote and maintained throughout embryonic development. The imprint maintenance is particularly important in pre-implantation embryos because it has to operate against the wave of genome-wide epigenetic reprogramming. First, the oocyte-specific isoform of the maintenance methyltransferase DNMT1, called DNMT1o, maintains the imprints at one single cell cycle in pre-implantation development [48]. Thus, the embryos derived from the oocytes lacking DNMT1o exhibited loss of DNA methylation at the germ-line DMRs and altered expression of the associated imprinted genes in about half of the cells [48]. More recently, it was reported that the zygotically expressed, somatic form of DNMT1, called DNMT1s, maintains the methylation imprints at the other cell cycles of pre-implantation development [49, 50]. At present we do not know how the DNMT1 isoforms specifically find the DMRs among many other DNA regions, but a recent study suggested that a mammalian-specific region near the amino terminus of DNMT1 is probably involved in the discrimination [51].

Other than DNMT1, the following proteins may also have a role in the imprint

maintenance in early embryos. First, ZFP57, an oocyte protein required for *de novo* DNA methylation of the *Snrpn* DMR, was shown to be present as a maternal protein in early embryos and essential for the maintenance of DNA methylation at several paternally and maternally methylated germ-line DMRs [46]. Second, PGC7 (also called Stella), another maternal protein, was shown to protect some germ-line DMRs from being reprogrammed in pre-implantation embryos [52], but how this multi-domain protein achieve this is unknown. Third, a methyl-CpG binding protein MBD3 has a role in maintaining the paternal methylation imprint at the *H19* DMR in pre-implantation embryos [53]. This appears to involve the recruitment of the Mi-2/NuRD repression complex to the highly CpG-methylated paternal allele of the *H19* DMR. However, an MBD3 depletion did not affect other imprinted genes examined and therefore the involvement of this protein seems locus-specific.

After implantation, the maintenance of the imprints requires DNMT1s in somatic lineages [14]. In addition to DNA methylation, however, the DMRs are also marked by differential histone modifications: the less CpG-methylated allele is marked by H3K4me and histone acetylation while the more CpG-methylated allele is marked by H3K9me3, H4K20me3 and H2A/H4R3me2 [54, 55]. Interestingly, DNA methylation seems less important for the imprint maintenance in the trophoblast (placenta). This was first demonstrated at the *Ascl2* (also called *Mash2*) locus: in the trophoblast lacking DNMT1, the maternal-specific expression of *Ascl2* was maintained [56]. Later, it was shown that mutations in *Dnmt1* do not cause loss of imprinting of the placenta-specific genes in an imprinted cluster on mouse chromosome 7 [57]. Here, the silent paternal alleles were marked by repressive histone modifications such as H3K9me2, mediated by G9a, and H3K27me3, mediated by the Polycomb repressive complex 2

(PRC2) [57, 58]. Indeed, mice lacking G9a lose the mono-allelic expression patterns of the placenta-specific genes [59]. Also, in embryos lacking Eed, a component of the PRC2 complex, a subset of the paternally repressed genes was aberrantly activated in the trophoblast [60]. These observations highlight the importance of histone modifications in the imprint maintenance, but whether these marks are also present in germ cells and/or gametes (especially in oocytes) is yet to be seen.

Mechanism of imprint erasure in primordial germ cells

The erasure of the imprints in PGCs is most likely reflected by DNA demethylation. It can occur in an active or a passive way, but the rapid DNA demethylation in PGCs suggests that it might be an active process [33]. Although there are various possible mechanisms for active DNA demethylation [61], recent studies have provided clues to the demethylating mechanism in PGCs. The activation-induced cytidine deaminase (AID), which is expressed in tissues where demethylation occurs, was shown to be capable of deaminating 5-methylcytosine (5mC) to thymidine (T) in DNA [62]. The resulting T-G mismatches might trigger a DNA repair pathway and result in a loss of 5mC. Both genome-wide and locus-specific analyses on AID-deficient PGCs demonstrated that AID contributes to global demethylation, and also demethylation at some imprinted DMRs (*H19* and *Kcnq1ot1*) in both male and female PGCs [63]. Nevertheless, considerable DNA demethylation still occurs in PGCs deficient for AID, indicating the presence of other demethylation mechanisms.

The ten-eleven translocation family proteins (TET1, TET2 and TET3) catalyze the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) *in vitro* and *in vivo* [64, 65]. 5hmC

may facilitate passive DNA demethylation by excluding the proteins involved in maintenance methylation such as DNMT1 [66] or may represent an intermediate in an active demethylation pathway [61]. Since TET1 and TET2 are significantly expressed in PGCs at E11.5 and E12.5 [67], when the imprinted DMRs undergo demethylation, it is possible that the TET family proteins play a role in the erasure of the imprints. For example, if 5hmC is recognized by a glycosylase, then the base excision repair (BER) pathway may restore the unmethylated state, as DNA demethylation in the PGCs is accompanied by the appearance of single-stranded DNA breaks and the activation of the BER components [67]. Further studies are needed to fully understand the precise mechanism of DNA demethylation and the imprint erasure in PGCs.

Genomic imprinting and cell reprogramming technology

Recent advancement in the cell reprogramming technology showed that somatic cell nuclei of differentiated states can be reprogrammed to a pluripotential state either by nuclear transfer or by using defined factors [68, 69]. In such a reprogramming process, pluripotential genes, developmental genes and tissue-specific genes are reprogrammed, but the parental-origin-specific epigenetic imprints, which ensure the mono-allelic expression of the imprinted genes, need to be maintained (Figure 1). It is unknown how the imprints at the DMRs escape from the global reprogramming, but errors in the imprint maintenance could be related to a reduced pluripotency, which is one of the major obstacles in iPS cell research.

Recently, it was reported that the expression state of the imprinted *Dlk1-Dio3* cluster on mouse chromosome 12 is often altered in iPS cells and can be used as a marker to evaluate the pluripotency [70]. In the affected iPS cell clones, a few imprinted genes, such as *Gtl2*, within

the *Dlk1-Dio3* cluster were abnormally silenced. Furthermore, these iPS cell clones contributed poorly to chimaeras and failed to support the development of entirely iPS cell-derived mice, whereas embryos derived from iPS cell clones with normal expression of these genes developed well [70]. The abnormalities at the *Dlk1-Dio3* cluster were not seen in embryonic stem (ES) cells. In the iPS cell clones with silenced *Gtl2*, DNA hyper-methylation and histone hypo-acethylation were detected at the DMRs within the cluster. Since these DMRs are normally methylated only on the paternal chromosome [71], the observed abnormalities are viewed as a "paternalization" of the maternal chromosome. In other words, the unmethylated state of the maternally derived DMRs was not maintained. At present, the precise cause of this aberrant silencing is unknown, but the reprogramming procedure itself seems to induce these epigenetic changes [70]. Since the aberrant silencing of the *Dlk1-Dio3* cluster is not frequent in cloned mice produced by nuclear transfer, the oocyte cytoplasm may contain a factor that protects the DMRs of this cluster from *de novo* DNA methylation. Clone-specific variations in the stability of mono-allelic expression of the imprinted genes were also reported in human iPS cells, but in this case various genes were affected (for example, *H19* and *KCNQ1OT1*) [72].

Outlook

Genomic imprinting is an excellent model system to study nuclear reprogramming in mammals because the epigenetic imprints regarding the parental origin are fully reprogrammed in each generation. In the last 10 years or so, many factors involved in each step of the imprinting cycle were identified, and we started to learn how this interesting phenomenon occurs. Nevertheless, there remain many unanswered questions, for example, how the

regulatory factors identify specific targets for imprint establishment in the germ-line, how the imprints escape from genome-wide reprogramming in pre-implantation embryos, and how the imprints are erased in PGCs. Furthermore, an interesting link between the epigenetic aberrations in imprinted gene clusters and reduced developmental potential has been discovered in mouse iPS cells. Thus the studies on the mechanisms underlying any step of the life cycle of the genomic imprints should contribute to the improvement of the reprogramming technology for animal cloning and iPS cell generation.

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Figure legends

Figure 1 Life cycle of the genomic imprints. The paternal (blue) and maternal imprints (red) are established in the germ-line and maintained through fertilization and subsequent embryonic development. However, the imprints are erased in PGCs before the new imprints are set. The imprints need to be maintained during the extensive reprogramming that occurs in animal cloning and iPS cell generation (blue arrow).

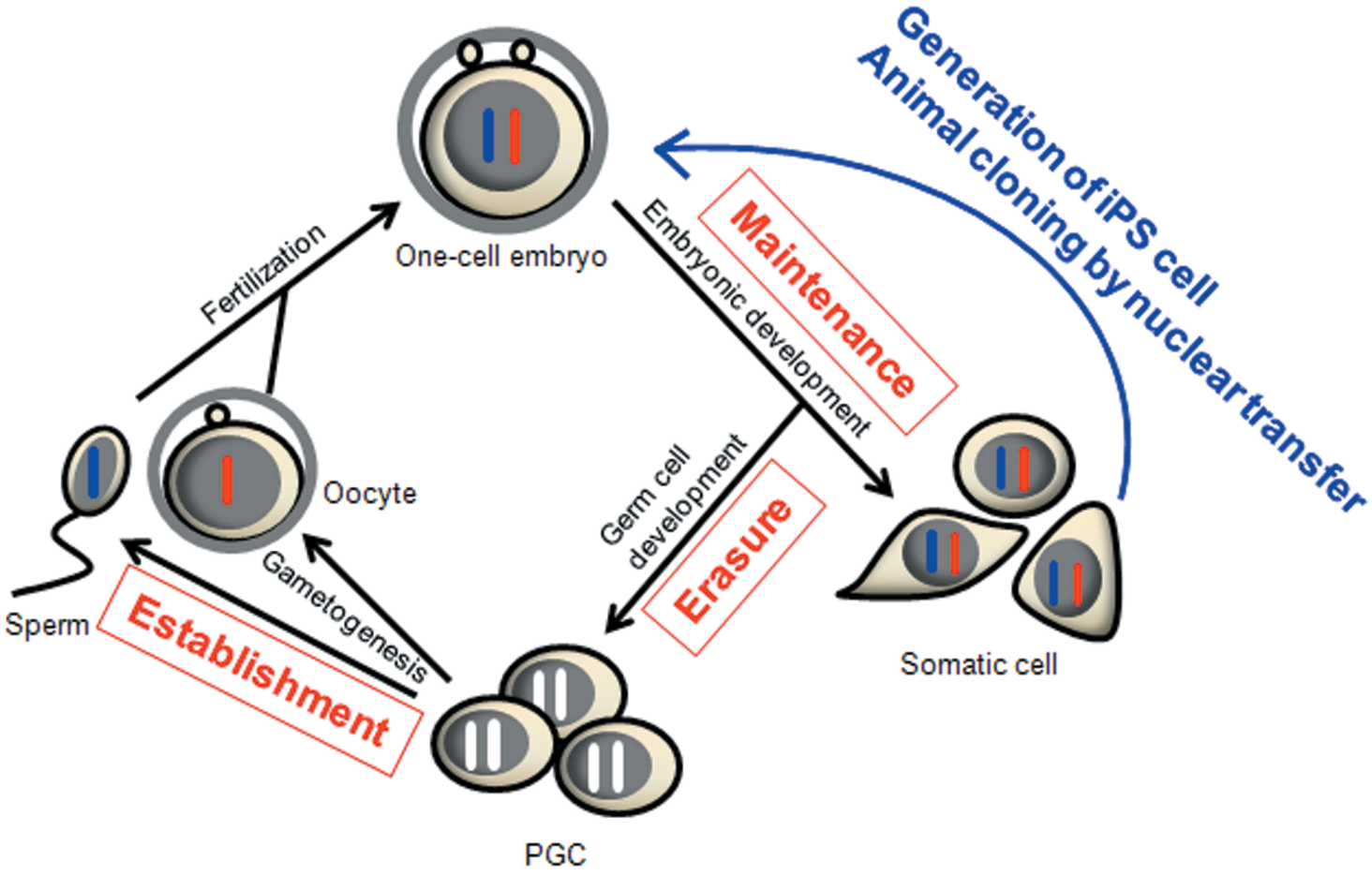
Generation of iPS cell
Animal cloning by nuclear transfer
One-cell embryo
Fertilization
Embryonic development
Maintenance
Oocyte
Sperm
Germ cell development
Erasure
Gametogenesis
Establishment
Somatic cell
PGC

Figure 1

Table 1 Factors involved in the life cycle of the genomic imprints

Step of the cycle	Factor name	Biochemical function	Germ-line DMRs affected in knockout		Reference
			Paternally methylated	Maternally methylated	
Establishment	DNMT3A	*de novo* DNA methyltransferase	*H19, Dlk1-Gtl2, Zdbf2, Rasgrf1*	*Snrpn, Peg1* (*Mest*), *Peg3, Igf2r*	Hata *et al*. 2002 Kaneda *et al*. 2004 Kato *et al*. 2007 Hiura *et al*. 2010 Kaneda *et al*. 2010
	DNMT3B		*Rasgrf1*		Kato *et al*. 2007 Kaneda *et al*. 2004 Kaneda *et al*. 2010
	DNMT3L	Cofactor of DNMT3A and DNMT3B	*H19, Dlk1-Gtl2, Rasgrf1*	*Snrpn, Peg1, Peg3, Igf2r*	Bourc'his *et al*. 2001 Bourc'his *et al*. 2004 Kaneda *et al*. 2004 Kato *et al*. 2007 Kaneda *et al*. 2010
	KDM1B	Histone H3K4 demethylase		*Peg1, Grb10, Zac1* (*Plagl1*), *Impact*	Ciccone *et al*. 2009
	ZFP57	KRAB zinc finger protein		*Snrpn*	Li *et al*. 2008
Maintenance	DNMT1 (DNMT1o and DNMT1s)	Maintenance DNA methyltransferase	*H19, Rasgrf1*	*Igf2r, Peg3, Snrpn*	Li *et al*. 1993 Howell *et al*. 2001 Kurihara *et al*. 2008 Hirasawa *et al*. 2008
	ZFP57	KRAB zinc finger protein	*Dlk1-Gtl2*	*Snrpn, Peg1, Peg3, Nnat*	Li *et al*. 2008
	PGC7 (Stella)	Maternal factor with a SAP domain and a splicing factor-like motif	*H19, Rasgrf1*	*Peg1, Peg3, Peg10*	Nakamura *et al*. 2007
	MBD3	Methyl CpG binding protein	*H19*		Reese *et al*. 2007
Erasure	AID	Cytidine deaminase	*H19*	*Kcnq1ot1* (*Lit1*)	Popp *et al*. 2010
	APE1*, XRCC1* and PARP1*	Base excision repair	Not analyzed	Not analyzed	Hajkova *et al.* 2010
	TET1* and TET2*	Conversion of 5mC to 5hmC	Not analyzed	Not analyzed	Hajkova *et al*. 2010 Ito *et al.* 2010

Asterisks (*) show that these factors are possibly involved.