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Keeping active endogenous retroviral-like elements in check: the epigenetic perspective

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Abstract. Endogenous retrovirus-like elements, or ERVs, are an abundant component of all eukaryotic genomes. Their transcriptional and retrotranspositional activities have great potential for deleterious effects on gene expression. Consequences of such activity may include germline mutagenesis and cancerous transformation. As a result, mammalian genomes have evolved means of counteracting ERV transcription and mobilization. In this review, we discuss epigenetic mechanisms of ERV and LTR retrotransposon control during mouse development, focusing on involvement of DNA methylation, histone modifications, small RNAs and their interaction with one another. We also address relevance of research performed in the mouse system to human and challenges associated with studying repetitive families. (Part of a Multi-author Review)

Keywords. LTR retrotransposon, epigenetics, DNA methylation, chromatin, mouse development, retroviral silencing, RNAi.

Introduction

It is well appreciated that most of the immediate effects of novel endogenous retroviral (ERV) integrations are either neutral, with no functional impact on the organism, or are harmful. New insertions taking place in germ cells result in germline mutations [1, 2], while such occurrences in somatic cells may cause cancerous transformations [3, 4]. Due to the typical germ cell-specific expression pattern that ERVs often display [5–7], new integrations in somatic cells are thought to be rare and can occur only if their transcription has been activated by, for example, demethylation, which often takes place on a global scale in transformed cells [8, 9]. Somatic insertions, some of which may target proto-oncogenes [3], can compromise genome integrity and play a role in malignant transformation. There is extensive evidence of cancerous transformation accompanied by aberrant expression of retrotransposons or ERVs in somatic cells of both human and mouse [3, 10–12], (also see a report in this issue). Moreover, changes in ERV expression have been documented in autoimmune diseases [13, 14]. Reports of intracisternal A particle (IAP)-induced aberrant transcripts in tissues of aging mice indicate that other processes besides cancer-induced genome-wide hypomethylation, such as normal aging, may result in ERV deregulation [15, 16].

Given the potential for harmful effects, it is therefore not surprising that eukaryotic genomes have evolved multiple lines of defense against active exogenous and endogenous retroviruses (see [17] for review). Indeed, ERV transcription is restricted in most differentiated tissues of animals and plants due to silencing directed by DNA methylation, histone modifications and RNA interference [18, 19]. The exact mechanisms of ERV silencing remain to be elucidated, and it is still unclear why some species show higher ERV activity than others. As we discuss further below, expression of ERV families, silenced in differentiated tissues, is

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detectable in germ cells, preimplantation embryos and placenta [5, 6, 20–22] despite many host surveillance mechanisms targeting ERVs during gametogenesis and early development. This is likely due to the fact that the regulatory sequences of ERVs are adapted to expression in these particular stages which allow transmission across generations. This strategy employed by ERVs is similar to the strategy of true transposons with exclusively vertical transmission that are mainly active in the germline, and is clearly different from the strategy of exogenous retroviruses, transmitted between individuals and thus readily infecting and replicating in somatic cells.

In this article, we review transcriptional and post-transcriptional mechanisms of ERV silencing, focusing on mouse germline and early development. However, it should be noted that the mechanisms described in this review are neither specific to rodents nor do they target exclusively ERVs. Their general principles are conserved across species and are aimed at protecting host genomes against deleterious effects of transposition.

Overview of epigenetic mechanisms

DNA methylation and DNA methyltransferases

It has been proposed [23] that the primary role of DNA methylation may in fact be host defense against transposable elements, and, specifically, endogenous retroviruses. The authors speculate that gene regulation and X-inactivation are secondary adaptations, as some species successfully regulate their gene expression and compensate for X-chromosome imbalance in the absence of DNA methylation [24]. Later reviews also favor the idea that gene silencing originated as a means of suppressing parasitic sequences [19]. In addition to DNA methylation acting at the level of transcriptional repression, a second line of defense involving RNA degradation is acting at the post-transcriptional level. In fact, the two can be interrelated. As discussed further below, double-stranded RNAs (dsRNAs) derived from retrotransposons may be capable of inducing both transcript degradation and DNA methylation via RNA interference (RNAi) pathways.

Among the numerous mechanisms of epigenetic silencing in mammals, DNA methylation is the most well studied. In the mammalian genome, a methyl group is predominantly targeted to the cytosine in the context of a CpG dinucleotide. Four enzymatically functional DNA methyltransferases (Dnmts), Dnmt1 [25], Dnmt3a, Dnmt3b [26] and Dnmt2 [27] have been identified in mammals (reviewed in [28]). All of these enzymes contain highly conserved DNA methyltransferase motifs but have different functional properties with respect to the substrate. Dnmt1 is considered to be the major maintenance Dnmt that functions primarily during DNA replication [29]. Dnmt3a and Dnmt3b are viewed as *de novo* Dnmts and are able to methylate both unmethylated and hemi-methylated DNA. Dnmt3l is a Dnmt-like protein with no enzymatic activity, which, however, is necessary for Dnmt3a and Dnmt3b to realize their full methyltransferase potential [30] (reviewed below). Dnmt2, possessing an extremely low level of Dnmt activity [31, 32], seems so play a role in RNA methylation [33]. Consistent with this fact, *Dnmt2*$^{-/-}$ zebrafish have reduced RNA methylation levels accompanied by developmental defects [34]. The exact functional niche of this methyltransferase in mammals remains to be determined.

Histone remodeling and histone methyltransferase*s*

In eukaryotic cells, the basic unit of chromatin is a nucleosome consisting of 146 bp of DNA wrapped around two tetramers of core histone proteins H3, H4, H2A and H2B. Histone core and histones tails, especially those of histones H3 and H4, are subject to covalent post-transcriptional modifications, particularly at their lysine and arginine residues. Among others, the most common and well-studied ones are methylation and acetylation, frequently associated with transcriptional control or localization to specific genomic neighborhoods [35]. Moreover, most of these modifications are cross-regulated. While some histone modifications are mutually exclusive, others promote deposition of different histone marks on other amino acid residues of the same histone tail [36].

Histone H3 tail at lysine 9 (H3K9) can be mono-, di- and trimethylated. In animals, H3K9 trimethylation has been implicated in silencing of repetitive elements [37, 38]. At least five H3K9-specific histone methyltransferases (HMTases) that deposit H3K9 methylation marks have been identified in mammals: Suv39 h1, Suv39 h2, Eset/SETDB1, GLP/Eu-HMTase1 and G9a/EuHMTase2 [39]. However, their niches in regulation of genes and repetitive elements are only beginning to be understood. Histone methylation and deacetylation may also be dictated by DNA methylation, likely promoting further reduction in active and increase in repressive chromatin marks [40–43] seen on repetitive elements.

Dynamics of epigenetic modifications in retroviral silencing

Much of our basic knowledge of ERV suppression originates from work investigating retroviral and lentiviral vector silencing. Embryonic stem (ES) cells provide a good model for studying silencing of

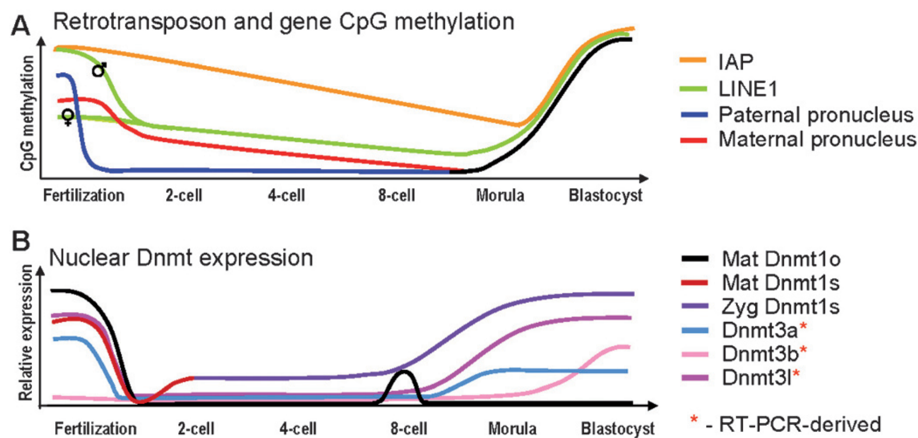

Figure 1. Epigenetic reprogramming in preimplantation development. (*A*) DNA methylation of retrotransposons and genes. Relative methylation status of IAP and LINE1 retrotransposons and maternal and paternal pronuclei. LINE1 methylation, different between maternal and paternal genomes, is indicated with respective signs. (*B*) Relative expression of Dnmts in the nucleus. Unless otherwise noted, expression levels are derived from immunofluorescence experiments. See text for references.

endogenous and introduced proviruses due to a high level of *de novo* methyltransferase activity [44]. Introduced proviruses are rapidly silenced in ES cells, suggesting that active epigenetic mechanisms of host defense, such as those depositing repressive chromatin modifications and establishing DNA methylation patterns, are in place (reviewed in [45]).

Transcriptional silencing of most retroviruses occurs within 2–3 days after infection [26, 46] regardless of the presence of *de novo* DNA methyltransferases [26, 47, 48]. However, DNA methylation is detected only 8–10 days post infection [26, 46, 49], suggesting a secondary role in retroviral silencing. After transgene integration, a rapid decrease in acetylation of histones H4 and even more so of H3 is observed, correlating with near complete silencing of expression by day 5 [50]. Substantial H3K9 dimethylation (H3K9me2) and DNA methylation of the transgene promoter and transcription start site are only evident at day 19, long after transgene is silenced, increasing progressively until days 70–90 [50]. These data suggest that H3K9 methylation and, even more so, DNA methylation lie downstream of deacetylation and transcriptional silencing, and that deacetylated histones may be a requirement for histone and DNA methylation to take place.

Regulation of ERVs in preimplantation development

Most work dedicated to methyltransferase function has been performed in ES, embryonic carcinoma (EC) cells or preimplantation mouse embryos, as they possess very high levels of *de novo* methylation activity. Notably, proviruses introduced before implantation are silenced and methylated in the adult, while those introduced at post-implantation stages remain unmethylated [51, 52]. The outcome of these experiments suggests that long-term silencing, irreproducible in the ES cell system, can be established only during early embryonic development.

Epigenetic reprogramming and ERV regulation in preimplantation development

Mammalian development is tightly associated with changes in global and local DNA and chromatin modifications. After fertilization, the paternal pronucleus undergoes rapid active, and the maternal pronucleus, slow passive demethylation, followed by passive demethylation of both genomes to their lowest level in the morula [53, 54]. The initiation of *de novo* methylation by Dnmt3a and Dnmt3b, which are upregulated at this time (Fig. 1B) [55–57], coincides with the onset of differentiation and results in hypermethylation of the inner cell mass (Fig. 1A) which gives rise to all tissues of the adult and from which ES cells are derived [53, 54]. The maternal pronucleus seems to have a high level of H3K9me2 and a low level of H4 acetylation, while the reverse is true for the paternal pronucleus, once protamines associated with sperm DNA are substituted for histones. Afterwards, both marks stay at a similar level until the blastocyst stage, with H4 acetylation peaking at the 8-cell stage [58, 59].

The kinetics of IAP methylation in early development were thoroughly examined in a study by Lane et al. [60], who showed resistance of this ERV family to demethylation throughout all stages of development. A high level of IAP methylation of approximately 95–98 % in mature sperm and oocytes remains at the same level in the zygote and decreases to 62 % by the blastocyst stage [60], staying considerably higher than the genome average [53, 54]. Surprisingly, the long interspersed nuclear element 1 (LINE1) non-LTR retrotransposon family of repetitive elements, though highly methylated in sperm, undergoes much more extensive demethylation after fertilization (Fig. 1A). It is therefore tempting to speculate that IAP elements

and possibly other ERVs may carry sequence elements or histone modifications similar to those of imprinted genes, preventing their demethylation in the zygote.

Recently, a PGC7/Stella protein, expressed in primordial germ cells, mature oocytes and preimplantation embryos, was implicated in protection of maternal pronucleus from rapid demethylation after fertilization. This protein also plays a significant role in maintaining DNA methylation on paternally imprinted genes and ERVs, such as IAPs, in the zygote. Preimplantation embryos derived from *Stella*$^{-/-}$ oocytes exhibit an over twofold reduction in IAP methylation levels [61].

Role of Dnmt1 proteins in ERV regulation

DNA methylation and Dnmt family members appear to be critically important, albeit to different degrees, for maintenance of genome integrity and regulation of repetitive elements, including ERVs. In addition to genome-wide demethylation, mouse embryos and/or ES cells deficient for Dnmt1 demonstrate demethylation of endogenous Murine leukemia virus (MLV) [26, 44], IAP [26, 62], MaLR [63] and MusD ERVs [Maksakova and Mager, unpublished results] as well as LINE1 retrotransposons [63] and major [26] and minor satellite repeats [44]. Moreover, IAPs are transcriptionally activated in response to demethylation [62]. Not unexpectedly, *Dnmt1*$^{-/-}$ embryos die before E9.5. While their maintenance DNA methylation is compromised, *Dnmt1*$^{-/-}$ ES cells efficiently methylate newly integrated MLV proviruses [26, 44] and successfully remethylate endogenous MaLR elements after transient exposure to demethylating agent 5-azaCytidine [63].

Several isoforms of Dnmt1, transcribed from alternative promoters, are expressed at different stages of germ cell and embryonic development [64, 65]. Dnmt1o, a shorter oocyte-produced isoform of Dnmt1 [65], was thought to be the only form of the maintenance Dnmt in cleavage-stage embryos, where the protein, retained in the cytoplasm, is functionally inactive. Dnmt1o may help maintain methylation patterns of imprinted *loci* when it enters the nucleus at the 8-cell embryonic stage (Fig. 1B) [66]. However, the mechanisms and functional significance of this translocation require further examination (reviewed in [67]), as does its correlation with transient increase in H4 acetylation levels. Since DNA methylation levels of satellite and retroviral IAP elements are similar between wild-type and heterozygous embryos derived from *Dnmt1o*$^{-/-}$ oocytes [66], the main role of Dnmt1o probably lies solely in maintenance of DNA methylation on imprinted genes.

The longer Dnmt1 isoform typically found in somatic cells, Dnmt1s, was initially undetected during early embryogenesis [68]. However, recent findings demonstrate that Dnmt1s is present in the nucleus of preimplantation embryo cells until the blastocyst stage [69, 70] (Fig. 1B). It is tempting to speculate that Dnmt1s may be responsible for maintaining DNA methylation on genomic sequences resistant to demethylation during preimplantation development, such as IAP ERVs and parentally imprinted genes. Most intriguingly, inactivation of Dnmt1s in the early embryo using either a Dnmt1s-specific neutralizing antibody or an RNAi approach results in partial demethylation of IAP LTRs compared with wild type at the morula stage [69], confirming that Dnmt1s plays a role in ERV suppression by maintaining DNA methylation during the wave of global demethylation occurring in the preimplantation embryo. The authors suggest that observed incompleteness of demethylation may have resulted from only partial inactivation of Dnmt1s with the methods used in the study or compensation by Dnmt1o, transiently present in the nucleus during the 8-cell stage.

Role of Dnmt3 proteins in ERV regulation

ES cells deficient for only one of the *de novo* methyltransferases, either Dnmt3a or Dnmt3b, are phenotypically normal and exhibit normal methylation levels of endogenous [26, Maksakova and Mager, unpublished results] and exogenous [26] proviruses. In contrast, *Dnmt3b*$^{-/-}$ embryos fail to develop to term and display slight demethylation of IAP and MLV ERVs accompanied by multiple developmental defects. *Dnmt3a*$^{-/-}$ mice have normal levels of ERV methylation and develop to term but die before 4 weeks of age [26]. Presumably, *de novo* methyltransferases Dnmt3a and Dnmt3b function in redundant pathways in ES cells but not during development [26]. Substrate specificity of each of the *de novo* Dnmts remains controversial: in one study, Dnmt3b but not Dnmt3a was shown to be partially responsible for methylation of MLV and a subset of centromeric minor satellite repeats in ES cells and embryos [26]. However, according to other reports, Dnmt3b1 exhibits preference for minor satellite repeats, while Dnmt3a and Dnmt3a2 splice isoforms are more efficient in methylating MLV and IAP retroviral sequences [71]. There is also some evidence that Dnmt3a and 3b may assist in maintenance of ERV methylation in ES cells [63].

Double *Dnmt3a*$^{-/-}$*3b*$^{-/-}$ embryos have a severe phenotype resembling *Dnmt1*$^{-/-}$. They lack somites and do not undergo embryonic turning, indicative of developmental arrest shortly after gastrulation. Embryos and/or ES cells deficient for both *de novo*

Dnmts, *Dnmt3a* and *Dnmt3b,* exhibit slight demethylation of IAP, MLV [26] and MusD elements [Maksakova and Mager, unpublished results]. However, there is a pronounced effect on previously unmethylated sequences in ES cells: introduced proviruses remain almost completely unmethylated [26], and endogenous MaLR elements are not re-methylated to normal levels following 5-azaCytidine-induced demethylation [63]. Dnmt3a and Dnmt3b levels are high in ES cells but drop upon differentiation, with somatic tissues expressing these enzymes at a very low level. Not surprisingly, newly introduced MLV proviruses remain unmethylated in fibroblast NIH3T3 cells which have reduced *de novo* Dnmt activity [26]. Interestingly, prolonged culturing of $Dnmt3a^{-/-}3b^{-/-}$, but not wild-type or $Dnmt3a^{-/-}$ or $Dnmt3b^{-/-}$ single-mutant ES cells causes a dramatic reduction in DNA methylation of both repetitive, such as IAP, MLV and minor satellite, and unique sequences [71], indicating that Dnmt1 alone is not sufficient for stable inheritance of DNA methylation in ES cells. This may explain low DNA methylation levels, similar to those in $Dnmt1^{-/-}$ cells [26, 37], of IAP LTRs in $Dnmt3a^{-/-}3b^{-/-}$ ES cells observed in some studies [37].

Dnmts were shown to be associated with histone binding protein 1 (HP1), a component of heterochromatin present in mammalian cells in three functional isoforms: α, β and γ [72, 73]. HP1 proteins favor binding to H3K9me2-associated DNA [74]. While all HP1 isoforms can interact with all Dnmts, this interaction can stimulate catalytic activity of only Dnmt1, resulting in higher local DNA methylation levels [74]. Presumably, HP1 interacts with H3K9me2-modified DNA and increases the enzymatic activity of the Dnmt1 it recruits, promoting faster and more efficient DNA methylation of the modified chromatin template. Dnmt1 can in turn stabilize the binding of HP1 to chromatin, facilitating formation and spreading of heterochromatin [72].

Role of histone modifications in ERV regulation

While H3K9 methylation has been implicated in ERV silencing, there is conflicting evidence on the role of different H3K9-specific HMTases in ERV suppression. According to some reports, DNA methylation levels of endogenous MLV [75] and IAP elements [M. Lorincz, personal communication] are similar to wild-type in Suv39 h1/2 double-KO (knockout) ES cells. Conversely, others detect increased IAP expression in Suv39 h1/2-deficient ES cells [37]. While SETDB1 is required for viability of ES cells, no IAP demethylation is observed in SETDB1 KO blastocysts [76]. Interestingly, IAP, MLV and MusD retroviral elements are significantly demethylated but are not transcriptionally induced in G9a KO ES cells [M. Lorincz, personal communication], suggesting involvement of multi-step complex mechanisms and possibly yet unidentified histone methyltransferases in ERV silencing. While the roles of HMTases in ERV regulation during embryonic development were not addressed or were inconclusive, embryonic lethality of HMTase-deficient mice [77] confirms the critical importance of these proteins in development.

Several genome-wide, as well as sequence-specific, analyses of histone modifications associated with ERV sequences have been performed in recent years. A study by Mikkelsen et al. reported that H3K9me3 and H4K20me3 marks, both associated with silencing, showed nearly identical patterns of enrichment on IAP ERVs [38] in ES cells. Work by Martens et al. identified H4K20me3 as the only mark on IAP LTRs that was significantly enriched above background [37]. This discrepancy may be due to the difference in sequence analyzed by the two groups. While Martens et al. amplified immunoprecipitated DNA with primer pairs specific for the IAP LTR promoter region and an internal *gag* region, Mikkelsen et al. sequenced and annotated all of immunoprecipitated DNA. The difference in the results may be explained by the fact that H3K9me3 modification is associated with the region not analyzed by Martens et al., or that only a subset of copies, not detected by specific primers, carries this mark.

The strongest association of H3K9me3 and H4K20me3 was detected for LTR retrotransposons, as well as telomeric and satellite repeats [38]. Intriguingly, this study showed that, of all ERV families in the mouse genome, the majority of the H3K9me3 mark in ES cells is associated with the most transcriptionally and retrotranspositionally active elements, IAP and ETn/MusD retrotransposon families [1]. Such selective targeting of active elements may result from homology-dependent RNAi-mediated silencing via targeting of repressive chromatin modifications, similar to mechanisms reported in yeast, plants and *Drosophila* [78]. Antisense ERV RNAs are produced in considerable amounts in ES cells [20, 21] and may potentially form double-stranded RNAs triggering RNAi. Importantly, 78 % of unique sequences marked with H3K9me3 and H4K20me3 were located within 2 kb of an LTR or satellite repeat [38], providing evidence of repressive chromatin spreading from retroviral sequences into proximal regions, which could lead to silencing of genes located in the vicinity of ERVs. However, specific examples of such an ERV-induced gene-silencing phenomenon have not been demonstrated in mouse.

Unexpectedly, despite nearly complete demethylation of IAP LTRs in $Dnmt1^{-/-}$ ES cell line [37] associated with massive transcriptional up-regulation [26, 62], no

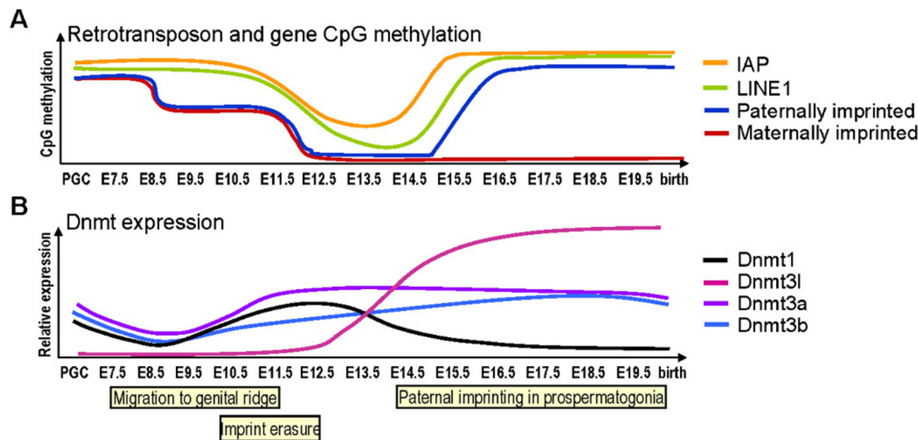

Figure 2. Epigenetic reprogramming in germ cell development. (*A*) DNA methylation of retrotransposons and genes. Relative methylation status of IAP and LINE1 retrotransposons and maternally and paternally imprinted genes. (*B*) Relative expression of Dnmts. See text for references.

reduction in the repressive H4K20me3 mark was observed in these mutants [37]. These results suggest either that efficient IAP transcription does not require unmethylated H4K20 or, alternatively, that only a few IAP elements lacking H4K20me3 and H3K9me3 modifications and located in permissive *loci* are highly expressed.

One of the most interesting observations from studies by Mikkelsen et al. and Martens et al. stems from analysis of chromatin marks associated with IAP LTRs in different cell lines. IAP transcription is low in mouse embryonic fibroblast (MEF) and trophoblast stem (TS) cell lines [37], correlating, surprisingly, with lack of repressive chromatin modifications on IAP elements in MEF, TS [37] and neural progenitor cells (NPCs) [38]. These observations suggest that in fully differentiated cell lines, lack of ERV expression is due to either stable DNA methylation or lack of transcription factors. In this case, repressive chromatin is not required to maintain lack of ERV transcription, as we have also found for MusD/ETnII elements in fibroblast versus ES cell lines [Maksakova and Mager, unpublished results].

In contrast to MEF or TS cells, repressive chromatin modifications associated with IAP LTRs are significantly elevated in retinoic acid (RA)-differentiated ES cells where IAP expression is also down-regulated [37]. This may reflect the dynamic nature of the differentiation process, with chromatin remodeling being the first step in ERV suppression only later followed by stable repression via DNA methylation [45]. During the process of differentiation, global chromatin changes toward suppression take place, silencing stem cell-specific genes and resulting in increase of repressive chromatin marks throughout repetitive satellite and interspersed elements. As RA-treated cells move further along the differentiation path and acquire gene expression patterns characteristic of differentiated cells, it is possible that their chromatin state will resemble that of MEF or TS cell lines.

Regulation of ERVs in germ cell development

Epigenetic reprogramming in germ cell development

Both male and female primordial germ cells (PGCs) arise in a posterior primitive streak of an embryo at E7.5 and start migrating to a genital ridge, entering it around E11.5 and continuing to divide mitotically until about E13.5 (reviewed in [54]). Upon their arrival at a genital ridge, PGCs are subject to extensive epigenetic reprogramming, resulting in erasure of parental imprints. This is manifested by rapid demethylation of maternally and paternally imprinted *loci* (Fig. 2A) and is presumed to be an active process (reviewed in [79]). In mouse, male and female gonads are morphologically indistinguishable at E12.5. Male germ cells undergo mitotic arrest at E13.5 in G1 phase. They resume mitosis just after birth, and the first spermatogenic stages enter meiosis at least a week after birth. Female germ cells undergo one more round of DNA replication and enter meiosis. They are arrested after birth in prophase of meiosis I which resumes only before ovulation [80]. In male germ cells, prospermatogonia, paternal imprints are established between E14.5 and birth, while in the female germ cells, maternal imprints are established after birth during oocyte growth [79] (Fig. 2A). H3K9me2 levels drop during germ cell migration [81, 82], consistent with decrease in levels of two major euchromatic H3K9 HMTases, G9a and especially GLP [83] and correlating with global genome demethylation. Following DNA demethylation and loss of H3K9me2, another repressive mark, H3K27me3, is up-regulated [81, 82] and probably functions in maintenance of gene silencing during the period of demethylation.

ERV regulation during normal germ cell development

The ability of IAP and, to a lesser extent, LINE1 retrotransposons to escape demethylation occurring in the preimplantation embryo and germ cells [84, 85] places these parasitic sequences on par with parentally imprinted genes, whose methylation is faithfully maintained after fertilization during global demethylation of the early embryo [86, 87]. According to bisulfite analysis of male germ cells [88], DNA methylation level of all examined genes and repetitive sequences is at its lowest in the E12.5 prospermatogonia. Even at that time, endogenous IAP ERVs are demethylated to only 45–50%. In E13.5 PGCs, IAPs maintain methylation level of 32%, while LINE1 DNA methylation level reaches 13% [60]. Subsequently, re-establishment of methylation, virtually complete by the time of birth, takes place on IAP and LINE1 elements [88] (Fig. 2A).

Intriguingly, transgenic mice with an IAP LTR driving expression of a reporter gene indicated that IAP promoter activity is restricted exclusively to undifferentiated spermatogonia in testis of late-stage embryos (E16.0) or adults and not detected in 2-cell embryos, blastocysts, E13.0 embryos or female germ cells [5]. Methylation patterns of the transgene LTR and endogenous IAP LTRs were identical, characterized by slight reduction in DNA methylation in spermatogonia compared with other organs, suggesting that the transgenic IAP LTR may be an appropriate simulation of endogenous IAP elements. Such restricted expression pattern suggests that either IAPs transcriptionally active in other tissues and developmental stages, such as thymus [89, 90] or early embryo [21, 91], possess regulatory elements absent from the LTR used in the transgene, or that a limited number of elements active in those tissues evade host suppression due to position effects [5].

Role of Dnmt3l in ERV regulation during germ cell development

Dnmt3l, a Dnmt-like protein lacking methyltransferase activity, is first detectable in both male and female germ cells around E12.5. In prospermatogonia, its level continuously increases until birth [92, 93] (Fig. 2B), concomitant with methylation of paternally imprinted genes. It is rapidly down-regulated after birth and disappears within 6 days, after most prospermatogonia have differentiated into dividing spermatogonial stem cells [92]. In the female germline, Dnmt3l is highly up-regulated only in growing oocytes after birth, when maternal imprints are set [93, 94]. Different promoters are responsible for Dnmt3l expression in oocyte and spermatogonia [95]. Dnmt3l is also present in ES cells and is down-regulated upon differentiation [96]. Interestingly, Dnmt3l is not essential for zygotic development, since *Dnmt3l*$^{-/-}$ embryos develop normally and have normal methylation levels of MLV ERVs and DMRs [96].

While non-essential for zygotic development, Dnmt3l is indispensable for maturation of both male and female germ cells. *Dnmt3l*$^{-/-}$ females, though fertile, produce embryos that die at E9.5 with neural tube defects and underdeveloped placenta [94, 96]. Embryos derived from *Dnmt3l*$^{-/-}$ oocytes display severe hypomethylation and aberrant expression of all maternally imprinted genes. Nevertheless, IAP retrotransposons maintain normal methylation levels, implicating Dnmt3l solely in establishment of maternal imprints during oocyte development [94, 96].

In the male, spermatogonia of *Dnmt3l*$^{-/-}$ mice fail to differentiate into spermatocytes, resulting in sterility of *Dnmt3l*$^{-/-}$ males. In contrast to *Dnmt3l*$^{-/-}$ female germ cells that have normal levels of IAP methylation, *Dnmt3l*$^{-/-}$ male germ cells display massive demethylation and transcriptional up-regulation of LTR- and non-LTR retrotransposons but not satellite repeats or paternally imprinted genes [92]. The fact that this effect is restricted only to spermatogonia and not somatic cells from embryonic testes indicates that Dnmt3l has a vital role in retrotransposon suppression in male germline [92]. While IAP elements resist erasure of imprints and maintain high DNA methylation levels in prospermatogonia of wild-type animals, Dnmt3l depletion results in dramatic drop of IAP methylation level to below 40% [88]. These results confirm that Dnmt3l, though lacking methyltransferase activity [97], plays a critical role in *de novo* methylation of retroviral elements in the developing male germline. Dnmt3l likely cooperates with Dnmt3a to establish methylation of maternally imprinted genes in female germ cells and retrotransposons in male germ cells [96]. Dnmt3l can stimulate *de novo* methylation activity of Dnmt3a but not Dnmt3b [98] by inducing a conformational change that facilitates Dnmt3a binding to DNA and a methyl group donor, *S*-adenosyl-L-methionine [30].

How can Dnmt3l recruit methyltransferase activity to repetitive sequences? Dnmt3l was shown to selectively recognize and bind nucleosomes containing unmethylated H3K4. It may then guide DNA methylation by recruiting and/or activating Dnmt3a [99]. The exact mechanism of Dnmt3l targeting to specific sequences requires further investigation. However, H3K4me2, found on unmethylated DMR alleles [100, 101], may be involved in protection of promoter regions from DNA methylation and repressive chromatin spreading [102]. Conversely, unmodified H3K4 is associated with methylated alleles of imprinted genes [100, 101]. Since Dnmt3l is capable of recognizing the absence of H3K4 methylation, it can recruit active DNA meth-

yltransferase Dnmt3a to unmethylated H3K4 to establish DNA methylation on imprinted genes and retrotransposons. Alternatively, H3K4me2 may protect unmethylated DMRs from DNA methylation by Dnmt3a-Dnmt3l complex. Moreover, Dnmt3l was shown to interact with histone deacetylase Hdac1 and contribute to DNA methylation-independent silencing [103]. As discussed later, Dnmt3l may be targeted to LTR-retrotransposons in the male germline via Piwi-interacting RNAs. However, the exact mechanism of Dnmt3l-mediated recognition of retrotransposon sequences directing them for suppression by Dnmt3a or DNA methylation-independent mechanisms is yet to be discovered.

Role of Dnmt1, Dnmt3a and Dnmt3b in ERV regulation during germ cell development

Dnmt1, Dnmt3a and Dnmt3b levels are low in E8.0 migrating germ cells, correlating with the first drop in genomic DNA methylation [81]. Afterwards, Dnmt3a and Dnmt3b are maintained at constant levels throughout germ cell development [93] (Fig. 2B).

Similar to ES cells and embryos deficient in only one of the *de novo* methyltransferases, IAP methylation is only slightly reduced in *Dnmt3b*$^{-/-}$ or *Dnmt3a*$^{-/-}$ single-mutant newborn prospermatogonia, suggesting that both methyltransferases function redundantly in the male germline [88]. As described above, *Dnmt3a*$^{-/-}$ and *Dnmt3b*$^{-/-}$ embryos die before producing mature germ cells, preventing the assessment of Dnmt3a and Dnmt3b roles in normal germ cell development. To counteract this problem, conditional KO technology was employed for disruption of *Dnmt3a* and *Dnmt3b* genes exclusively in germ cells of otherwise phenotypically normal animals [104]. While *Dnmt3b*$^{-/-}$ conditional mutants and their offspring show no apparent phenotype, offspring of *Dnmt3a*$^{-/-}$ conditional mutant females die at around E10.5, exhibiting complete lack of methylation at maternally imprinted *loci* and slight demethylation of IAP elements. *Dnmt3a*$^{-/-}$ conditional mutant males display impaired spermatogenesis and lack of methylation at some paternally imprinted *loci* in spermatogonia, while IAP methylation is unaffected [104].

The phenotype of both male and female germ cells deficient in Dnmt3a resembles that of *Dnmt3l*$^{-/-}$ germ cells [92, 96], confirming the critical role of Dnmt3l in stimulation of Dnmt3a-induced *de novo* methylation during germ cell development. Subsequent reports suggest that specifically Dnmt3a2, the shorter spliced isoform of Dnmt3a [71], is implicated in Dnmt3l-mediated regional DNA methylation in germ and ES cells [93, 105, 106]. Consistent with the view of Dnmt3l playing a crucial role in guiding *de novo* methylation established by Dnmt3a2 in the germline, Dnmt3l is the only methyltransferase substantially up-regulated during the only periods of *de novo* methylation in male (E17.5 testis) and female (adult ovary) germ cells [93].

Non-Dnmt proteins involved in ERV regulation

Role of Lsh1 in ERV regulation

Other proteins besides Dnmts have been implicated in ERV silencing. One of the most interesting ones is lymphoid-specific helicase 1 (Lsh1), originally identified in mouse fetal thymus tissue [107]. Lsh1 is a member of the SNF/SWI family of chromatin-remodeling proteins that use the energy of ATP hydrolysis to alter nucleosome structure [108]. These proteins are involved in modification of chromatin accessibility to DNA-binding proteins by facilitation of nucleosome sliding and displacement [108]. *Lsh1*$^{-/-}$ mice die within 24 h after birth with low birth weight, renal lesions and defects in lymphoid development [109]. They also display substantial reduction in genome-wide DNA methylation. However, localization of important markers of pericentric heterochromatin, such as HP1α and H3K9me3, is similar in wild-type and *Lsh1*$^{-/-}$ MEFs, at least at the resolution allowed by immunofluorescence. In addition, no difference is detected in H3K9me2 levels, a modification associated with silent euchromatic regions [110].

While association of IAP and LINE1 retrotransposons and satellite DNA with di- and trimethylated H3K9 was similar in *Lsh1*$^{-/-}$ and wild-type MEFs, the same repetitive sequences were enriched for di- and trimethylated H3K4 in *Lsh1*$^{-/-}$ MEFs, a hallmark of euchromatin and transcriptionally active regions [111]. Interestingly, none of the examined single-copy genes were affected, suggesting involvement of Lsh1 in repetitive element-specific H3K4 methylation. Intriguingly, Lsh1 deficiency leads to transcriptional activation specifically of IAP and major satellite, but not LINE1 sequences in MEFs and embryonic tissues [111, 112], prompting speculation that Lsh1 may have a predominant role in regulation of ERVs, as opposed to non-LTR retrotransposons.

Since histone methylation and acetylation are interrelated, the authors further examined H3 and H4 acetylation, also hallmarks of active chromatin. In *Lsh1*$^{-/-}$ embryos, satellite repeats and IAP retrotransposons display greater association with acetylated histones, while single-copy genes are not affected [112]. Genome-wide analysis of the transcriptionally perturbed sequences in brain and liver of *Lsh1*$^{-/-}$ embryos revealed that 80 % of all up-regulated transcripts contain transposable elements. Of these, 45 % contain LTR retrotransposon and 44 %, non-LTR

retrotransposon sequences [112]. Considering that LTR retrotransposons, or ERVs, occupy approximately 10% of the mouse genome *versus* 27% occupied by non-LTR retrotransposons [113], Lsh1-directed transcriptional repression preferentially targets ERVs compared with non-LTR retrotransposons. In addition, direct interaction of Lsh1 with satellite, non-LTR and LTR retrotransposon repetitive sequences but not single-copy genes was demonstrated [112].

What mechanisms can Lsh1 employ to regulate gene expression? As had been shown in MEF cells using episomal plasmids, Lsh1 plays a role in *de novo* DNA methylation and appears to enhance Dnmt3a and Dnmt3b-mediated DNA methylation and silencing [114]. In addition, Lsh1 has been shown to co-immunoprecipitate with Dnmt3a and Dnmt3b, but not Dnmt1, in nuclear extracts from EC and ES cells, emphasizing the role of Lsh1 in *de novo*, rather than maintenance, methylation. In human cells, HDAC1 and HDAC2 can be recruited to LSH1 through their interaction with DNMT1 which, in turn, binds DNMT3B [115]. Intriguingly, catalytic functions of DNMT3B and DNMT1 are not essential for LSH1-mediated suppression. Since transcriptional repression of the reporter is not immediately accompanied by DNA methylation [115], LSH1 may function primarily through establishment of inactive deacetylated chromatin via recruitment of HDACs, while DNA methylation is a secondary event resulting from high local concentration of DNMT3B and DNMT1. Based on its affinity for repetitive DNA, Lsh1 may function as a scaffolding protein to recruit Hdacs and Dnmts for epigenetic suppression of satellite repeats, non-LTR and, predominantly, LTR-retrotransposons, or ERVs.

Role of other proteins in ERV regulation

Other SWI/SNF chromatin-remodeling family proteins besides Lsh1 have been shown to bind de *novo* methyltransferases. A SWI/SNF protein Brg1 binds Dnmt3a in association with Hdac1 and a methyl-CpG binding protein MBD3a. Similar to Lsh-mediated silencing, catalytic activity of Dnmt3a is not required for suppression induced by this complex [116], suggesting the role of Dnmt3a may be limited to recruitment of other repressive proteins, such ac Hdac1 and Brg1, to DNA. Dnmt3a, in association with Hdac1, may also be recruited to DNA via interaction with DNA-binding transcriptional repressors, such as RP58 [117], to guide transcriptional suppression. Little is known about target specificity of these repressive proteins, and it remains to be seen whether any of them are involved in ERV regulation. Extensive analyses of retroviral expression in stem cells led to identification of factors that restrict retroviruses in this particular cell type. One such factor is TRIM28 (Kap1, TIF1-β), a co-suppressor involved in silencing through interaction with Kruppel-associated box zinc finger proteins. TRIM28 recognizes and associates with a primer-binding site of an MLV retrovirus, inducing binding by heterochromatin protein HP1γ and deposition of H3K9me2. These events result in HP1-dependent epigenetic silencing subsequently reinforced by DNA methylation [118, 119]. It remains to be determined whether TRIM28 or other suppressor proteins may guide transcriptional repression of other ERV families.

Uhrf1, a protein also known as Np95 in mouse and ICBP90 in human, was recently implicated in DNA methylation maintenance and retrotransposon silencing. *Uhrf1*$^{-/-}$ ES cells exhibit global genomic demethylation along with IAP and LINE1 demethylation [120, 121] and transcriptional de-repression [121] similar to that observed in *Dnmt1*$^{-/-}$ ES cells. Uhrf1 may regulate suppression by recruitment of Dnmt1 to sites of hemimethylated DNA [120] and di- and tri-methylated H3K9 [122]. In addition, Uhrf1 contributes to establishment and/or maintenance of Hp1α and H3K9me3 localization in interphase nuclei [122] and is able to recruit HDAC1 [123]. While mechanisms of Uhrf1 targeting are unclear, these characteristics make Uhrf1 an attractive candidate for playing a role in ERV silencing, heterochromatin establishment and spreading and faithful reproduction of DNA methylation during cell division.

An entirely different mechanism of retrotransposon silencing was recently described in fission yeast *Schizosaccharomyces pombe.* It involves DNA transposon-derived CENP-B proteins highly conserved in mammals and functioning in the process of centromere formation in yeast [124]. Of the three CENP-B homologues in yeast, Abp1, Cbh1 and Cbh2, the former two bind LTRs of *Tf2* and *Tf1* retrotransposons, and Abp1 alone is sufficient for their transcriptional repression. The silencing of LTR-retrotransposons is apparently induced through direct recruitment of Hdacs Clr3 and Clr6 bound to Abp1. The authors propose that CENP-B proteins also interact with each other, segregating retrotransposons into clusters termed Tf bodies. Such clustering may facilitate genome surveillance of retrotransposon transcription and illegitimate recombination via deacetylation or other mechanisms [124]. Further investigation is required to determine whether similar systems may be operating in mammalian genomes.

Regulation of LTR-retroelements by small RNAs

Suppression mechanisms reviewed above fall into the category of transcriptional silencing imposed by DNA methylation or histone modifications. Another mechanism of retrotransposon suppression requires mediation by small RNAs. There are several regulatory pathways involving small RNAs that have diverse targets and different levels of regulatory activity. MicroRNAs regulate genes at post-transcriptional and translational levels, while small interfering RNA (siRNA) and Piwi-interacting RNA (piRNA) defend the genome against exogenous and endogenous parasitic elements at transcriptional and post-transcriptional levels. In this review, we are focusing mainly on the reported cases of LTR-retrotransposon silencing *via* small RNA molecules at both transcriptional and post-transcriptional levels.

Regulation of LTR-retroelements by siRNA

RNA interference is a regulatory mechanism initiated by siRNAs through cleavage of dsRNA molecules. The enzyme Dicer, responsible for dsRNA processing, generates 21–23 nucleotide long siRNA duplexes. Dicer is also involved in biogenesis of microRNAs, small RNAs which are involved in gene regulation and are thus not included in this review. siRNA duplexes are loaded onto the RNA-induced Silencing Complex (RISC). The catalytic component of RISC is an Argonaute protein with a slicer activity directed against the target mRNA molecule bearing a perfect sequence match to the siRNA. mRNAs containing sequences complementary to the original dsRNA are degraded in a process known as PTGS (post-transcriptional gene silencing). The siRNA pathway is conserved in most species where it acts mainly as a defense mechanism against viruses with a dsRNA stage in their lifecycle or against artificially introduced dsRNA. However, siRNA of endogenous origin is a frequent phenomenon in plants and fungi, contributing significantly to transposon silencing (reviewed in [125–127]). In animals, very recent evidence of such endogenous siRNAs in *Drosophila* (not reviewed here) and in mouse has been reported.

The formation of dsRNA requires transcription of both strands. In the case of ERVs, this may occur due to presence of active antisense promoters either downstream of the insertion site or contained within the ERV itself. The latter is illustrated by LTR-retrotransposon *micropia* in *Drosophila,* shown to produce anti-sense mRNA [128] and IAP elements that have LTRs with both sense and antisense promoter activity [129].

Dicer-dependent small RNAs corresponding to ERVs have also been described in the mouse. Both sense and anti-sense transcripts of MuERV-L and IAP elements are present in 2-cell and 8-cell embryos, and depletion of Dicer leads to a 50 % increase in expression of both ERV families in 8-cell embryos [21]. In ES cells, an additional type of small RNA corresponding to repeats was characterized [130]. These small RNAs are of the same size as siRNAs but are independent of Dicer function. Among them, one particularly abundant Dicer-independent small RNA corresponds to the ETn/MusD family known to be transcriptionally active during early embryogenesis [22, 131, 132], though significance of this finding is not yet clear. This small RNA is antisense to the primer-binding site of ETn/MusD, and thus it cannot be excluded that it is a degradation product of the tRNA that serves as a primer during reverse transcription of these elements, rather than a regulatory RNA.

Small RNAs corresponding to repeats have also been detected in the oocyte, their size of 21–23 nt and enrichment in A and U residues at their 5′ ends suggesting they are siRNAs [133–135]. Repeats represented by these siRNAs contain IAP, MT and LINE1 retrotransposon sequences. Indeed, introduction of sense and anti-sense sequences of IAP, MTA and LINE1 elements into an EGFP reporter gene in the fully grown oocyte results in degradation of EGFP mRNA, indicating that endogenous siRNAs target repeat-derived sequences [134]. Interestingly, mapping of these siRNAs revealed that the sequences they are derived from cluster in distinct genomic locations [133, 135]. Some of these locations also produce piRNAs in the oocyte, as will be detailed below. Interestingly, not only MT LTR retrotransposons, but also a large number of host genes are up-regulated in Dicer-deficient oocytes. Surprisingly, a highly significant proportion of these genes show no complementarity to any known microRNA as would be expected for typical Dicer-dependent gene regulation. In fact, these genes bear repeats in their 3′ untranslated regions (UTRs), suggesting that in female germline, host genes may be regulated by RNAi via their transposon-related sequences [136].

Regulation of LTR-retroelements by Piwi-interacting RNA (piRNA)

Recently, a new small RNA pathway, mainly directed against transposons, was described in animals. As their name indicates, piRNAs bind a particular subfamily of Argonauts, the Piwi-like proteins specifically expressed in germ cells, in accordance with the hypothesis that their main role is defense of the genome against transposition [137]. The Dicer-independent biogenesis of piRNAs results in small RNAs of 24–30 nt, longer than the small RNAs processed by Dicer. The precursor molecule of piRNAs is not dsRNAs, but

probably long RNA transcripts encoded by specific loci in the genome [138, 139]. These loci, referred to as piRNA clusters [140], are rich in transposable element (TE) fragments inserted in both orientations. Although the exact mechanisms for biogenesis of the piRNAs remain unknown, the wealth of data provided by *Drosophila* studies lead to the so-called ping-pong model that is discussed below in more detail.

In *Drosophila*, there are three Piwi-like proteins (Piwi, Aubergine and Ago3) expressed specifically in male and female germlines. An exception is Piwi, also expressed in somatic cells in contact with the ovary [141, 142]. About three-quarters of *Drosophila* piRNAs bound to the three Piwi-like proteins in the ovary correspond to repeat-associated siRNAs (rasiRNAs) [140]. They are produced by ~142 piRNA clusters located in pericentromeric or telomeric heterochromatin, although the majority of piRNAs are accounted for by only ~15 clusters. Each cluster gives rise to piRNAs corresponding to both strands, suggesting that clusters are transcribed in both orientations with only a few being unidirectionally transcribed. Piwi and Aubergine preferentially bind piRNAs corresponding to the minus strand of active TEs, while Ago3 shows the opposite bias [140, 141]. This information, combined with a 10 nucleotide long sequence complementarity of the sense and antisense piRNAs lead to the ping-pong model for piRNA biogenesis. In this model, antisense piRNAs are loaded onto Piwi or Aubergine proteins to guide the cleavage of sense mRNAs encoded by active TEs scattered throughout the genome, thus generating sense piRNAs. The sense piRNAs are loaded onto Ago3 and in turn guide the cleavage of antisense piRNA cluster transcripts. This mechanism leads to a feedback loop in which target molecules produce more regulatory molecules that can degrade more targets [140, 141]. In agreement with this model, all three Piwi-like proteins were shown to possess slicer activity [141, 142].

Confirmation of the repressive role of piRNAs towards TE activity came from a large piRNA locus mapped to the same genomic region as the long-known *flamenco* locus. *Flamenco* is located in pericentromeric heterochromatin of the X chromosome and is known to repress transposition of *gypsy, ZAM* and *Idefix* LTR-retrotransposons [143–145]. In accordance with the piRNA-cluster nature of *flamenco*, its ability to repress *gypsy* is abolished in *Piwi* mutants [146]. Furthermore, in *flamenco* mutants, the amount of piRNAs produced by this locus is substantially decreased, and the level of *gypsy* mRNAs is increased 20-fold [140]. In fact, a very broad range of *Drosophila* TEs seem to have corresponding piRNAs, and consequently a variety of LTR-retrotransposons seem to be regulated by this pathway. For example, Saito et al. reported piRNAs corresponding to 21 different types of LTR-retrotransposons [142]. Moreover, expression of LTR retrotransposons such as *roo* and *mdg1* is elevated in the piRNA biogenesis pathway mutants [138].

Another animal model in which piRNAs have been studied is zebrafish. It has two Piwi-like proteins, Ziwi and Zili. Ziwi is expressed in male and female gonads and early embryos [147]. Small RNAs of the size characteristic for piRNAs were identified in the germline, their sequences indicating that they also originate from piRNA clusters distributed widely throughout the genome. Unlike *Drosophila* piRNAs, only one-third of the zebrafish small RNAs in the ovary and one-quarter in testes correspond to transposons; the rest correspond to simple repeats or intergenic regions. However, 60 % of rasiRNAs in the zebrafish are derived from LTR-retrotransposons, while LTR-retrotransposons account for only 8 % of TEs in the genome, suggesting that these elements are specifically regulated by the piRNA pathway.

In the mouse, three Piwi-like proteins, Miwi, Mili and Miwi2, are expressed in the germline in different developmental stages [148–150] (Fig. 3). Mutation of these genes impairs spermatogenesis but no phenotype has been reported for female mutants. Ten-to-fourteen-day old *Mili*$^{-/-}$ and *Miwi2*$^{-/-}$ males show a significant increase in IAP and L1 element expression [137, 149, 151]. In male germline, piRNAs have been cloned from fetal [151], pre-pachytene [137, 152] and pachytene [139, 152] stages. However, only those purified from fetal and pre-pachytene germ cells are repeat-rich [151, 153] (Fig. 3). In fact, piRNAs from different stages of spermatogenesis are not transcribed from the same clusters, resulting in different compositions of the piRNA population. Among pre-pachytene piRNAs bound to Mili, 35 % are rasiRNAs, the rest matching mainly to non-annotated regions. Among rasiRNAs, 34 % correspond to LTR elements. This proportion is higher in fetal testis, with 55 % of rasiRNAs being derived from ERVs [151]. In female germline, both endogenous siRNAs and piRNAs corresponding to repeats were cloned from fully grown oocytes [133, 135]. Sixty-two percent of piRNAs are rasiRNAs, and among these, approximately one-third are derived from ERVs, mainly MT and IAP [133]. These piRNAs are bound to the Mili protein, the only murine Piwi-like protein that is expressed in oocytes [135]. Around two-thirds of the repeat-derived fraction of siRNAs correspond to ERVs and are particularly enriched in MT elements. As may be expected, oocyte piRNAs are also produced by genomic clusters, but unlike *Drosophila* or mouse testis, some of the oocyte-specific clusters are also a source of endogenous siRNAs [133, 135]. Thus, con-

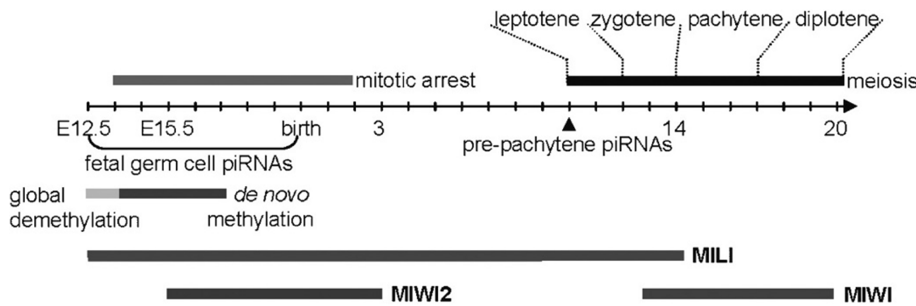

Figure 3. Expression of Piwi-like genes during mouse spermatogenesis. Spermatogenesis from embryonic day 12.5 (E12.5) to day 20 after birth is represented in the diagram. The most important events concerning cell division, DNA methylation and Piwi-like gene expression are depicted. Stages profiling repeat-rich piRNAs are also indicated (see text).

trol of transposition in female germline seems to depend on both siRNA and piRNA pathways, possibly explaining the lack of phenotype in female *Piwi* mutants [133]. However, it seems that the two pathways have preferential targets, MT and RLTR10 being mostly regulated by siRNAs, and IAP by piRNAs, as suggested by mutants of the respective pathways [135]. In human and rat, piRNAs have also been isolated from testes, revealing that while large piRNA clusters are syntenic between the three mammalian species, the smaller clusters are predominantly unique [139].

Targeting of transcriptional silencing to LTR-retroelements by piRNAs

Until recently, the mechanisms by which DNA methylation and other chromatin modifications target repeats in animals remained largely unknown. However, it is becoming increasingly clear that similarly to yeast and plants, animal small RNAs may guide these repressive mechanisms to silence repeats at the transcriptional level through their nucleotide complementarily. More precisely, the Piwi pathway seems to be involved not only in degradation of repetitive mRNA, but also in DNA methylation and recruitment of heterochromatin proteins in the germline.

In *Drosophila*, Piwi pathway mutants display delocalization of heterochromatin proteins HP1 and HP2 and reduction of H3K9 methylation [154]. Furthermore, Piwi protein may bind HP1α and co-localize with it on polytene chromosomes. This binding is RNA-dependent [155]. This opens the possibility that Piwi, guided by a complementary piRNA, binds DNA and initiates heterochromatinization and its spreading by recruitment of HP1α and histone methyltransferases. However, the effect of Piwi on chromatin is dependent on genomic context, since it can have an activating role in transcription of heterochromatic piRNAs [156]. Consistent with the role for piRNAs in transcriptional silencing of TEs, *Piwi-like* gene mutants lacking rasiRNAs show reduced levels of heterochromatin marks on TEs such as *I* element and *copia,* concomitant with increase in their expression [157].

In mouse, *Mili* and *Miwi2* homozygous mutants have reduced IAP and LINE1 DNA methylation levels compared with heterozygous animals [149, 151, 153]. Interestingly, *Mili*$^{-/-}$ and *Mili*$^{+/-}$ fetal germ cells display similar LINE1 and IAP methylation levels during global demethylation. However, during the process of *de novo* methylation, LINE1 and IAP methylation is reduced by half in homozygotes compared to heterozygotes [151]. This observation suggests that Mili is involved in *de novo* methylation of repeats in fetal testes. Moreover, as discussed above, the same kind of impaired DNA methylation is observed in *Dnmt3l* mutants, raising the possibility that Mili guides Dnmt3l to repeats for *de novo* methylation. While the role of piRNAs in DNA methylation is suggested by *Mili* and *Miwi2* mutants, more evidence is needed to unravel the exact mechanism of this phenomenon. Interestingly, microRNAs have been recently reported to control *de novo* methylation of gene promoters, indicating that other classes of small RNAs can direct transcriptional gene silencing [158, 159].

Regulation of genes by ERVs and epigenetic mechanisms

As discussed above, host organisms have evolved several epigenetic mechanisms to repress transposon activity. Nonetheless, it is also becoming clear that particular copies of ERVs can be co-opted to regulate host genes. For example, several LTR-derived gene promoters have been described in human [160, 161, 162]. These phenomena, extensively discussed in other reviews [163, 164] and detailed below, may also involve epigenetic mechanisms and may shed light on different aspects of ERV domestication.

ERVs and gene regulation during development

As mentioned previously, ERVs are particularly prone to expression during early mouse embryogenesis. Such examples include ETn/MusD [22], MuERV-L [21, 165, 166] and IAP retrotransposons [21, 91]. Thus, it seems reasonable to predict that not only

ERVs but also genes promoted by ERVs would be transcribed predominantly in this timeframe. Indeed, a case of special interest in accordance with this prediction is the MT subfamily of MaLR retrotransposons. This subfamily accounts for 13% of all transcripts in the fully grown oocyte [20], although it comprises less than 5% of the genome [113]. In the 2-cell embryo, however, the majority of transposon transcripts are encoded by another family of ERVs, MuERV-L. Interestingly, in addition to LTR retrotransposon transcripts, many gene transcripts initiating in the LTR of MTs are present in the fully grown oocyte, while LTRs of MuERV-Ls are more prominent in promoting transcripts in the 2-cell embryo. This suggests a correlation between expression of ERV copies promoting genes and general expression of the ERV family these specific copies belong to [20]. Furthermore, such chimeric transcripts, found in the fully grown oocyte and preimplantation embryo, are not detectable in other developmental stages or adult tissues [20]. It is tempting to speculate that LTR-promoted genes are regulated by the same epigenetic mechanisms as the corresponding ERVs. Indeed, siRNAs with complementarity to MTs are abundant in the oocyte [133–135], whereas MuERV-L derived siRNAs were characterized in the 2- and 8-cell stage embryo [21], correlating nicely with the expression profile of these families as well as that of chimeric transcripts they promote [20]. In accordance with this hypothesis, impairment of the siRNA pathway in oocytes up-regulates not only MT retrotransposons, but also a large number of host genes harboring MTs in their 3′UTRs [136].

In line with the observations described above, a large proportion of domesticated ERVs are active in placenta, a tissue highly permissive for ERV expression [167–169]. These domestications relate either to placental proteins encoded by ERVs, as described for primates [170], mouse [174] and sheep [171], or LTR-derived gene promoters specifically active in placenta reported in human [160, 162, 164]. In fact, slightly lower DNA methylation levels in placenta compared with somatic adult tissues [172, 173] may account for the overall higher expression of ERVs and may in part explain the frequent occurrence of domesticated ERVs in placenta. However, it seems that human LTR-derived gene promoters specifically active in placenta are completely unmethylated in this tissue. Conversely, random copies of the same ERV family have diverse methylation levels which, however, are always higher than those of their gene-promoting counterparts [172]. This suggests that human LTR-derived promoters domesticated millions of years ago differ in their methylation levels from the bulk of ERV copies and may be treated by the cell similarly to tissue-specific gene promoters. As discussed below, the vast majority of human ERVs are no longer active and thus may not be specifically targeted by the silencing machinery of the cell. In this respect, mouse and human may not be comparable, since the former needs to actively regulate ERVs while the latter may perceive its ERVs as intergenic sequences.

In addition to encoding proteins involved in placentation, such as syncytins [170] and Peg10 [174], ERVs may have a role in development. For example, it has been reported that reverse transcriptase, abundant in the zygote due to LINE1 and LTR retrotransposon activity, is essential for normal early embryonic development, its depletion resulting in developmental arrest at the 2- and 4-cell stages [175]. This data suggests that a certain level of ERV expression during preimplantation stages is required for normal development. In conclusion, whether and to what extent ERVs play a functional role in development remains speculative and awaits further experimental tests. However, it is plausible that early development and gametogenesis are the timeframes that domesticated ERVs are likely to operate in, given their expression during these particular stages.

Challenges of investigating epigenetics of repetitive families

Many groups estimate DNA methylation levels of different retrotransposon families based on bisulfite sequencing. This approach, however, should be treated with caution. Diverse variants of elements exist within each family making it almost impossible to capture all subpopulations only a few of which may be transcriptionally active. Moreover, many ERV families encompass hundreds or even thousands of elements located in different chromatin contexts. Thus, a typical selection of 10–20 clones amplified from bisulfite-treated DNA for each ERV family represents only a tip of the iceberg likely not representative of the whole population. For the purpose of whole-genome estimation of DNA methylation at repetitive elements, genomic Southern blotting remains the golden standard. Even then, the choice of probes may affect the results, since solitary LTRs frequently account for the bulk of retroviral sequence in the genome and may not represent methylation patterns characteristic of full-length ERVs. For the lack of better option, a combination of Southern blotting and bisulfite sequencing may provide a fine resolution view coupled with estimation of whole-genome methylation on repetitive sequences.

Another concern relates to transcriptional up-regulation of ERVs seen in some mutant mice and mutant

or tumor cell lines. Whether transcription is estimated by RT-PCR or Northern blotting, it is usually unclear if the detected transcripts are produced by multiple de-repressed copies or by just a few elements in a permissive genomic context. Indeed, mostly younger and recently integrated MT [176] and MusD [131] LTR retrotransposons are expressed in permissive cells, suggesting this trend may persist when ERVs are re-activated. Additionally, expression and demethylation of only select IAP types was detected in tumor cell lines [177, 178]. The only solution for determining if multiple or only a few select elements are transcribed is sequencing of RT-PCR products. Mapping the source elements of these transcripts, however, is challenging due to the high degree of sequence similarity among different copies, the high level of ERV polymorphisms between mouse strains [179] and the existence of only one reference genome, C57BL/6.

Is the epigenetic control of mouse ERVs relevant to human?

The mechanisms of epigenetic ERV regulation in humans remain poorly documented. Moreover, very little information is available on epigenetic development of human embryos and germ cells. Thus, the question remains whether results obtained from analysis of data on epigenetic ERV suppression in mouse is applicable to human. An important difference between the two species is that human ERVs, with a possible exception of HERV-K, are currently inactive, their fossil sequences as the only evidence of past activity. Despite detection of active transcription in multiple tissues and cell lines [180, 181] no new integration events have been observed for any human ERV. This is likely due to the history of modern humans, subjected to multiple evolutionary bottlenecks that may have eliminated master ERV copies by virtue of genetic drift. The current activity of HERV-K is quite controversial. Polymorphisms [10, 182], copies with identical 5′ and 3′ LTRs and purifying selection observed in their envelope genes argue in favor of current or at least recent activity of a few members of the HERV-K family [183–185]. However, it is clear that the vast majority of detectable ERVs in the human genome integrated from 5 to 80 million years ago and are no longer mobile [185]. Therefore, members of most ERV families in humans have diverged substantially from each other, making these sequences more comparable to unique intergenic or intronic DNA than to repeats. This divergence renders homology-dependent mechanisms of epigenetic silencing difficult to envisage. It seems unlikely that siRNAs or piRNAs target such divergent sequences since they require a high degree of complementarity to act. However, these mechanisms are very likely present in humans and act against transposition of active transposons such as L1 s and Alus. This idea is supported by the existence of piRNA clusters in humans [139] and by the silencing of L1 by siRNAs in cell lines [186]. Concerning DNA methylation of human ERVs, the very few existing studies suggest that DNA methylation is in accordance with the expression profile of specific copies [172, 187, 188], but the high diversity of methylation and the abundance of slightly methylated copies also suggest that this type of silencing is not specifically targeted to these copies but mainly depends on locus-specific factors.

Despite our increasing knowledge of mechanisms involved in ERV regulation and impact of ERVs on genomes of host species, many details are unclear and are yet to be discovered.

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