

L1 Regulation in Mouse and Human Germ Cells

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1 Introduction

Transposable elements (TEs) represent one of the most dynamic components of mammalian genomes. Genome sequencing has illuminated the abundance, complexity, and diversity of TEs. TEs constitute approximately 50 % of human and mouse genomes (Lander et al. 2001; Waterston et al. 2002). Based on the mode of mobilization, they are classified as either DNA transposons, which mobilize through a cut-paste mechanism, or retrotransposons, which mobilize through a copy-paste mechanism (i.e., via an RNA intermediate). While DNA transposons have lost their activities in the human and mouse genomes during evolution, retrotransposons have been far more successful in colonizing these genomes (Lander et al. 2001; Waterston et al. 2002). Retrotransposons are further categorized into LTR (long terminal repeat) retrotransposons and non-LTR retrotransposons. LTR retrotransposons are also known as endogenous retroviruses (ERVs) and comprise 9 % and 10 % of the human and mouse genomes, respectively (Lander et al. 2001; Waterston et al. 2002). ERVs remain active in rodents (Maksakova et al. 2006), but do not show any recent insertional activity within the human genome (Magiorkinis et al. 2015). Non-LTR retrotransposons include long interspersed elements (LINEs) and short interspersed elements (SINEs). LINE-1s (L1s) constitute 17 % and 19 % of the human and mouse

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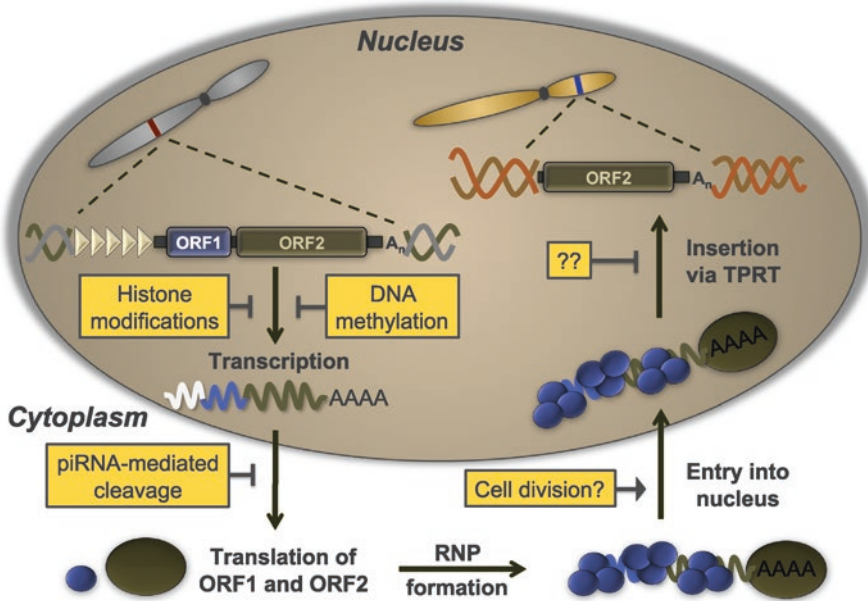


Fig. 1 L1 is regulated at distinct stages of replication. L1 mobilizes through a bicistronic mRNA intermediate and inserts into the genome through target primed reverse transcription (TPRT). L1s must overcome transcriptional silencing mechanisms. Both piRNA-guided DNA methylation and several histone marks (H3K9me3/2 and H2A/H4R3me2) suppress the expression of L1s in the germline. Posttranscriptionally L1s face (both pachytene and fetal) piRNA-mediated endonucleolytic cleavage. It remains unclear what barriers or host-factors are involved in nuclear import and TRPT

genomes, respectively (Lander et al. 2001; Waterston et al. 2002). A full-length L1 is ~6 kb in length and contains an internal promoter in its 5' untranslated region (5'UTR), two open reading frames (ORF1 and ORF2), and a poly(A) signal in its 3'UTR (Furano 2000) (Fig. 1). SINEs, on the other hand, do not encode any proteins and rely on L1 proteins for mobilization.

Both mouse and human genomes have over 500,000 copies of L1, most of which are incapable of further mobilization. The lack of activity is primarily due to "structural defects." In other words, mutations concurrent or subsequent to retrotransposition (i.e., 5' truncations, inversions, substitutions, INDELs, and any other types of debilitating mutations) render the elements incompetent (Szak et al. 2002). Within these inactive elements lie the remnants of many L1 families and subfamilies, highlighting the evolving battles between retrotransposons and the host (Khan et al. 2006). Only full-length intact L1s retain the ability to mobilize autonomously and amplify within the genome, and have the potential to generate additional full-length copies. It is estimated that the diploid human genome harbors an average of 80–100 retrotransposition competent L1 elements (Brouha et al. 2003). The number of

potentially active elements is much higher in the mouse genome (Goodier et al. 2001). It is worth noting that retrotransposition-competent L1s usually belong to younger L1 families and many are polymorphic among individuals. Accordingly, there can be large interindividual variation in genomic L1 retrotransposition potential (Beck et al. 2010).

Retrotransposons impact and modify the genome in a multitude of ways (Goodier and Kazazian 2008). The most obvious is insertional mutagenesis, which is highlighted by the observed >100 cases of human diseases caused by L1-mediated retrotransposition events (Hancks and Kazazian 2016). Thus, in the short term, retrotransposition activity represents a formidable threat to the host genome and must be properly regulated in both somatic and germ cells. However, in the long term, TEs may benefit a species through expansion of genomic material and alteration of expression patterns. Since L1s mobilize through an RNA intermediate, it expands the repertoire of alterations that L1s can engender to the genome. Due to its weak polyadenylation signal, donor L1s frequently utilize downstream polyadenylation signals and thus mobilize 3' flanking genetic information (3' transduction) (Moran et al. 1999). Intronic L1s can be exonized through cryptic splicing events (Zemojtel et al. 2007). Cellular mRNAs can also be mobilized, resulting in the expansion of processed pseudogenes (Esnault et al. 2000). In fact, through a process termed exaptation, TE-derived genes have adopted critical functions in many physiological processes, including immune response, centromere assembly, and placental formation (Alzohairy et al. 2013). Additionally, there is evidence that some TE sequences have been exapted into novel regulatory elements, potentially serving as promoters, enhancers, silencers, or insulators (Rebollo et al. 2012; de Souza et al. 2013). TEs may also spread repressive epigenetic marks to neighboring sequences and affect the activity of nearby promoters (Rebollo et al. 2011; Macfarlan et al. 2011; Grandi et al. 2015). Finally, L1 sequences may serve important roles during X chromosome inactivation through participating in the assembly and propagation of heterochromatin on the inactive X chromosome (Chow et al. 2010).

In order to survive or remain active in the genome, an L1 family needs to consistently generate full-length insertions that can be passed through the germline to the next generation, albeit expectedly at rates that do not significantly endanger the reproductive fitness of the host (Bestor 2003). Such insertions may occur by *bona fide* retrotransposition in developing germ cells, or by retrotransposition during early embryonic development in cells fated to become primordial germ cells (PGCs). The nature of TE regulation in the germline has been expounded in several recent reviews (Bao and Yan 2012; Zamudio and Bourc'his 2010). However, our understanding of just how complex the dance is between the two unwilling partners—host and L1s—has been expanded by new insights into the transcriptional and posttranscriptional coordination and the subtleties of regulatory mechanisms. This review will focus on retrotransposons, especially L1s, and how they are regulated in the mouse and human germlines. As LTR retrotransposons remain active in the mouse genome, differential regulation of L1 and LTR retrotransposons will be compared as appropriate. Our goal is to accent recent regulatory insights and provide a synopsis of the interplay between the host and L1.

2 L1 Activities Can Be Controlled at Different Stages of L1 Replication

L1 retrotransposition involves multiple steps, which can be schematically summarized as transcription, translation, ribonucleoprotein (RNP) formation, nuclear entry, and target-primed reverse transcription (Goodier and Kazazian 2008) (Fig. 1). It begins when a full-length donor L1 is transcribed into a bicistronic mRNA by RNA polymerase II. L1 mRNA is then exported to the cytoplasm, where the two L1 proteins are synthesized and form an RNP complex with the template L1 mRNA. The ORF1 protein (ORF1p) has nucleic acid binding and chaperone activities. Both activities are essential for retrotransposition (Kolosha and Martin 2003; Martin et al. 2005). The ORF2 protein (ORF2p) possesses two enzymatic activities: endonuclease and reverse transcriptase, both playing vital roles in target-primed reverse transcription (TPRT) of L1 mRNA (Feng et al. 1996; Mathias et al. 1991; Moran et al. 1996). To generate a new copy, the full-length donor L1 has to negotiate with the host cell through each of these steps. On the other hand, each step provides an opportunity for the host cell to control L1 activity.

From the L1's perspective, host regulatory mechanisms can be generally categorized as transcriptional regulation or posttranscriptional regulation. Inarguably, transcriptional regulation can be the most effective means of controlling L1 activities, simply because without transcription retrotransposition is impossible. *In vivo*, this has been illustrated by conditional activation of L1 transgenes in mice either through Cre-mediated excision of a transcriptional stop cassette (An et al. 2008) or through tetracycline induction of an inducible promoter (O'Donnell et al. 2013). Indeed, multiple mechanisms have been adopted to transcriptionally repress full-length L1s in the germline, including DNA methylation, histone modifications, and the fetal Piwi-interacting RNA (piRNA) pathway (Fig. 1). Posttranscriptional regulation includes both fetal and pachytene piRNA pathways. Additionally, entry into the nucleus and access to open chromatin seems to be an inherent obstacle for mobilization. While it may seem arbitrary to group regulatory methods into these categories, it highlights the multiple layers of regulation observed within the germ cells. Each of these regulatory mechanisms will be discussed in the context of germ cell development. Most of the mechanisms have been principally characterized in the mouse male germline (Sects. 4 and 5), but emerging factors involved in L1 regulation in the female mouse germline will be discussed (Sect. 6). L1 expression and regulation in human germ cells will also be summarized (Sect. 8). As this discussion occurs, keep in mind that essentially two antagonistic evolutionary processes are at play: the host needs to suppress mobile DNA from irreparable damage and TEs need to adapt and “reproduce” in the germline to survive.

3 L1 Expression During Male Germ Cell Development

Male germ cell development involves distinct cellular stages (Fig. 2). In the mouse, it starts from a founder population of PGCs at around embryonic day (E) 7.25 (Saitou and Yamaji 2012). Shortly after, they begin migration toward the future

gonadal site and colonize the genital ridge at ~E10.5. PGCs exit the mitotic cell cycle and arrest in G0 phase between E12.5 and 14.5 (McLaren 2001; Western et al. 2008), becoming prospermatogonia. The mitotically arrested prospermatogonia resume proliferation after birth at around postnatal day (P) 2, and give rise to undifferentiated and differentiating spermatogonia via mitotic divisions (Yoshida et al. 2006; de Rooij and Russell 2000; Drumond et al. 2011). The most primitive spermatogonia may undergo up to ten successive mitotic divisions before differentiating into preleptotene spermatocytes, starting at P8–P10, signaling the first wave of meiosis. Leptotene, zygotene, pachytene, and diplotene spermatocytes start to appear approximately at P10, P12, P14, and P17, respectively, during the prophase of meiosis I. Haploid spermatids first appear at P20 after two meiotic divisions and differentiate into mature spermatozoa at P30 (de Rooij and Russell 2000; Nebel et al. 1961; Bellve et al. 1977). Spermatogenesis continues in the adult testis in an

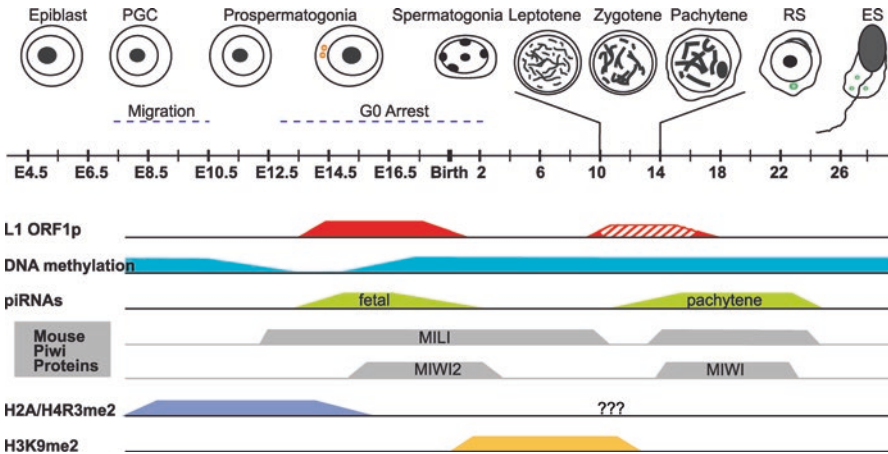


Fig. 2 L1 expression and important regulatory factors during male germ cell development. The timeline depicts male germ cell development in mice from PGC specification through the first wave of spermatogenesis (*RS* round spermatid, *ES* elongating spermatid). Normally suppressed through multiple transcriptional and posttranscriptional mechanisms, there are two periods of well-documented L1 expression. The first results from the loss of DNA methylation during fetal DNA reprogramming around E13.5–E14.5. DNA methylation is quickly restored, presumably guided to active TE elements through MIWI2-bound fetal piRNAs. H2A/H4R3me2 acts to suppress excessive L1 expression at this stage. This histone modification has also been found in post-natal germ cells but a stage-specific profile remains unavailable (indicated by *question marks*). The other period of L1 expression (*striped area*) seems to occur sporadically in a subset of spermatocytes. H3K9me2 is diminished at L1 loci during the transition from spermatogonia to spermatocytes. Additionally, there is extensive chromatin remodeling during meiosis, both of which likely contribute to the sporadic L1 expression observed in spermatocytes. Pachytene piRNAs associated with MIWI posttranscriptionally degrade TEs in pachytene spermatocytes and are known to contribute to the absence of L1 expression for the remainder of spermatogenesis. A role in the germline has not been established for zinc finger proteins (*box in dotted line*)

asynchronous yet precisely timed fashion: spermatogonia stem cells divide mitotically every 8.5 days and form spermatozoa 34.5 days later (Oakberg 1957).

In normal human and mouse individuals, L1 expression is predominantly found in germ cells (Rosser and An 2012). In the male germline, it is restricted to two specific developmental time windows (Fig. 2). The first time window occurs in the mitotically arrested prospermatogonia. Abundant ORF1p signals are detected in E14.5–E17.5 fetal testes (Trelogan and Martin 1995; Aravin et al. 2009). The second time window is in spermatocytes. Prominent ORF1p signals are observed in primary spermatocytes (leptotene and zygotene stages) of the prepubertal mouse testis (P10, P14, P18, and P25) (Branciforte and Martin 1994). In the adult testis, L1 ORF1p is detected sporadically (i.e., not in all cells or seminiferous tubules) by immunofluorescence in leptotene, zygotene, and pachytene spermatocytes (Soper et al. 2008), although positive signal was also reported in round spermatids, elongating spermatids and residual bodies when detected by immunohistochemistry in the initial study (Branciforte and Martin 1994). The same polyclonal antibody was used for both studies. The variation in ORF1p expression pattern might be due to methodological differences. Note in the following sections, when the term upregulation or derepression is used, it refers to a significant increase in L1 expression (i.e., above the normal levels) or unexpected expression in otherwise non-expressing cellular stages.

4 Transcriptional Silencing of L1s During Male Germ Cell Development

4.1 Transcriptional Silencing by DNA Methylation

DNA methylation is a critical mechanism for transcriptional silencing of retrotransposons in the germline. During animal development, there are two critical periods during which CpG methylation is programmatically removed from TEs: first in pre-implantation embryos and then in migrating/post-migratory PGCs (Messerschmidt et al. 2014). In particular, L1 5'UTR sequences undergo rapid demethylation in mouse PGCs between E10.5 and E13.5 (from 65 to 17%) (Lane et al. 2003; Hajkova et al. 2002; Ohno et al. 2013), and later become fully remethylated, through de novo methylation, in prospermatogonia by E17.5 (Lees-Murdock et al. 2003). This demethylation correlates with modest L1 expression in normal prospermatogonia (Fig. 2). As will be discussed in later sections, now we know that other regulatory mechanisms exist to prevent excessive L1 activities when L1s are hypomethylated.

The role of DNA methylation is clearly supported by TE activation in mice deficient in DNA methyltransferases (DNMTs). The mammalian genome encodes three enzymatically active cytosine methyltransferases (DNMT1, DNMT3A, and DNMT3B). The earliest in vivo evidence is from functional ablation of DNMT1. DNMT1 prefers hemi-methylated DNA as its substrate and mediates the mainte-

nance of genomic methylation patterns. Deletion of *Dnmt1* causes embryonic lethality in mice (Li et al. 1992). In somatic tissues of *Dnmt1*-deficient mouse embryos, there is widespread hypomethylation and derepression of a major class of mouse LTR retrotransposons, intracisternal A particles (IAPs) (Walsh et al. 1998). However, a change in methylation of other types of retrotransposons, including L1s, has not been reported in *Dnmt1*-deficient embryos. It is possible that L1 methylation may be unaffected because in other cellular environments, such as mouse embryonic stem cells (ESCs), DNMT1 appears to play a major role in regulating IAPs but not in regulating L1 expression (Li et al. 2015a). Indeed, unlike IAPs, L1 expression is undetectable in *Dnmt1*-deficient embryos (Bourc'his and Bestor 2004). UHRF1 (also known as Np95 and ICBP90) has an essential role in DNA methylation maintenance by recruiting DNMT1 to hemi-methylated DNA sites (Sharif et al. 2007; Bostick et al. 2007). Similar to *Dnmt1* knockouts, deletion of *Uhrf1* causes embryonic lethality. Interestingly, *Uhrf1* deletion is accompanied by demethylation and derepression of IAPs, L1s, and SINE-1 (Sharif et al. 2007).

DNMT3A and DNMT3B are required for *de novo* DNA methylation during animal development. Mice deficient for *Dnmt3a* die at ~4 weeks of age and those deficient for *Dnmt3b* die in utero (Okano et al. 1999). Embryos deficient for both *Dnmt3a* and *Dnmt3b*, but not for either enzyme alone, display reduced methylation at C-type retroviruses, IAP, and major satellite repeats, suggesting DNMT3A and DNMT3B have redundant functions at these sequences. In contrast, minor satellite repeats appear to be specific targets of DNMT3B (Okano et al. 1999). Detailed methylation analyses of conditional *Dnmt3a* and *Dnmt3b* knockouts revealed the timing and target specificity of DNMT3A and DNMT3B in the male germline (Kaneda et al. 2004; Kato et al. 2007). In terms of timing, the methylation of repetitive sequences and paternally differentially methylated regions (DMRs) occurs progressively in fetal prospermatogonia and is completed by the newborn stage (Kato et al. 2007). In terms of target specificity, DNMT3A mainly methylates B1 SINEs and some paternal DMRs, such as the H19 and Dlk1/Gtl2 loci. DNMT3B predominantly methylates minor and major satellite repeats. Both DNMT3A and DNMT3B are required for *de novo* methylation of L1 and IAPs as well as the paternal DMR at the Rasgrf1 locus (Kaneda et al. 2004; Kato et al. 2007).

DNMT3L, a homologue of DNMT3A/3B, lacks cytosine methyltransferase activity but is specifically expressed in growing oocytes of adult females and prospermatogonia of perinatal males (Bourc'his et al. 2001). Consistent with its germ cell specific expression pattern, deletion of *Dnmt3l* does not affect animal viability but causes infertility in both sexes (Bourc'his et al. 2001). In knockout males, spermatogenesis is arrested at the zygotene/early pachytene spermatocyte stages (Bourc'his and Bestor 2004; Webster et al. 2005). Despite the lack of enzymatic activity, DNMT3L plays a pivotal role in *de novo* methylation of all repetitive sequences and paternal/maternal DMRs examined in the germline (Bourc'his et al. 2001; Hata et al. 2002; Bourc'his and Bestor 2004; Webster et al. 2005; Kato et al. 2007). For example, deletion of *Dnmt3l* results in the global loss of *de novo* methylation at L1s and IAPs (Bourc'his and Bestor 2004; Kato et al. 2007) as well as B1 SINEs (Kato et al. 2007) in male germ cells. More significantly, hypomethylation

leads to TE derepression, as evidenced by high levels of L1 and IAP transcripts in neonatal and juvenile testes (Bourc'his and Bestor 2004). As compared to some piRNA pathway mutants discussed later, the spermatogenic defect manifested in *Dnmt3l* mutants appears to be more pronounced. Germ cells were completely absent in some seminiferous tubules by 4 weeks and in almost all tubules by 8–10 weeks (Hata et al. 2006). This Sertoli-cell-only phenotype suggests a functional deficit in spermatogonial stem cells. Indeed, careful analysis of neonatal animals revealed a mitotic defect in spermatogonia and reduced germ cell numbers as early as P6 (La Salle et al. 2007). In contrast, the female infertility has been attributed to the loss of maternal imprinting and inappropriate expression of maternally imprinted genes in fetuses (Bourc'his et al. 2001; Hata et al. 2002). Accordingly, IAPs and major satellite repeats show little change in methylation in fetuses carried by *Dnmt3l* knockout females (Bourc'his et al. 2001).

Barring a disruption of the de novo methylation process, the vast majority of the full-length L1 elements are remethylated by birth and remain hypermethylated as the germ cells progress through spermatogenesis (Fig. 2). So far there is no evidence for global or dynamic demethylation of L1s at the onset of meiosis (Oakes et al. 2007). However, not all copies of retrotransposons are methylated in spermatocytes (Molaro et al. 2014). A small fraction of L1s, SINEs, and LTR retrotransposons remain hypomethylated in adult spermatocytes as compared to E13.5 PGCs. These hypomethylated loci comprise both old and young subfamilies but are enriched for “middle-aged” subfamilies, such as the F subfamily of L1s and the MT subfamily of LTRs (Molaro et al. 2014). Such constitutively hypomethylated loci may underlie the sporadic L1 ORF1p expression seen in prepuberal and adult spermatocytes (Branciforte and Martin 1994; Soper et al. 2008) (Fig. 2), especially when considering the extensive chromatin remodeling during meiosis (Crichton et al. 2014).

4.2 The Role of Fetal piRNA Pathway in Transcriptional Silencing

piRNAs play critical roles in regulating retrotransposon expression. piRNAs are small noncoding RNAs abundant in germ cells. Unlike miRNAs (typically 19–22 nucleotides), piRNAs are longer and range from 24 to 31 nucleotides. First described in mouse, rat, and human adult testes in 2006 (Aravin et al. 2006; Grivna et al. 2006a; Girard et al. 2006; Lau et al. 2006), piRNAs are expressed mainly in two separate phases of male germ cell development. Accordingly, they are loosely categorized into fetal piRNAs and pachytene piRNAs (Fig. 2). The mouse genome encodes three Piwi proteins (MIWI/PIWIL1, MILI/PIWIL2, and MIWI2/PIWIL4). Fetal piRNAs are associated with MILI and MIWI2, highly enriched for TE sequences (Aravin et al. 2007; Kuramochi-Miyagawa et al. 2008), and function in both transcriptional and posttranscriptional silencing of retrotransposons (this section and Sect. 5.1). In contrast, pachytene piRNAs are associated with MILI and

MIWI, relatively depleted of TE sequences, expressed from genomic clusters (Aravin et al. 2006; Grivna et al. 2006a; Girard et al. 2006; Grivna et al. 2006b), and have an important role in posttranscriptional silencing (Sect. 5.2). More details on piRNA production and processing can be found in recent reviews (Iwasaki et al. 2015; Czech and Hannon 2016). Here we will focus our discussion of piRNAs as to what is applicable to TE regulation.

Transcriptional silencing by the fetal piRNA pathway is primarily mediated through DNA methylation. This putative mechanism is supported by molecular phenotypes in *Mili* and *Miwi2* knockout mice. Both *Mili* and *Miwi2* are expressed in embryonic testes when the genome is being remethylated (Kuramochi-Miyagawa et al. 2001) (Fig. 2). MILI expression starts at E12.5 in both sexes and stops after birth in female but continues until the round spermatid stage in males. In contrast, MIWI2 expression is restricted from E15.5 to P3 in male and absent in female germ cells (Aravin et al. 2008; Kuramochi-Miyagawa et al. 2001). Deletion of either *Mili* or *Miwi2* leads to male-specific infertility, mimicking the *Dnmt3l* knockout phenotype in spermatogenesis. Spermatogenesis arrests at the zygotene stage in *Miwi2* mutants (Carmell et al. 2007) and at the early pachytene stage in *Mili* mutants (Kuramochi-Miyagawa et al. 2004). The spermatogenic defect is accompanied by the concomitant loss of piRNAs and TE methylation (Aravin et al. 2008; Kuramochi-Miyagawa et al. 2008; Carmell et al. 2007). The role of the fetal piRNA pathway on DNA remethylation is highly specific towards repetitive sequences, as whole genome bisulfite sequencing shows that global methylation is not affected in *Mili* and *Miwi2* mutants (Manakov et al. 2015; Molaro et al. 2014). In agreement with the proposed pathway, deletion of *Dnmt3l* has no effect on piRNA production, confirming that the fetal piRNA pathway acts upstream of *de novo* DNA methylation (Aravin et al. 2008).

The fetal piRNA-mediated methylation appears to selectively target active full-length retrotransposons (Molaro et al. 2014). In spermatocytes, where genome-wide remethylation has already occurred, overall TE methylation levels are very similar between wild-type and *Mili*-deficient mice. This observation suggests that most TEs are remethylated nonselectively along with the rest of the genome by a default wave of *de novo* DNA methylation (Molaro et al. 2014). In contrast, the youngest L1 subfamilies (e.g., A and T) manifest the most pronounced reduction in methylation and corresponding upregulation in *Mili*-deficient spermatocytes (Molaro et al. 2014). A remarkable feature of this phenomenon is that it selectively targets transcriptional regulatory regions, i.e., L1 and IAP promoters are differentially methylated between wild-type and *Mili*-deficient backgrounds (Molaro et al. 2014). This provides a mechanism to effectively silence full-length, transcriptionally active elements. In conclusion, fetal piRNAs partner with the DNA methylation machinery to re-suppress L1s through *de novo* DNA methylation, specifically homing in on active full-length elements (Fig. 3).

The two principal components of the fetal piRNA pathway, MILI and MIWI2, cooperate but carry distinct roles in fetal germ cells. Besides temporal difference in expression, they differ in intracellular location (Aravin et al. 2008; Aravin et al. 2009). In male prospermatogonia, MILI is localized primarily in the cytoplasm, in

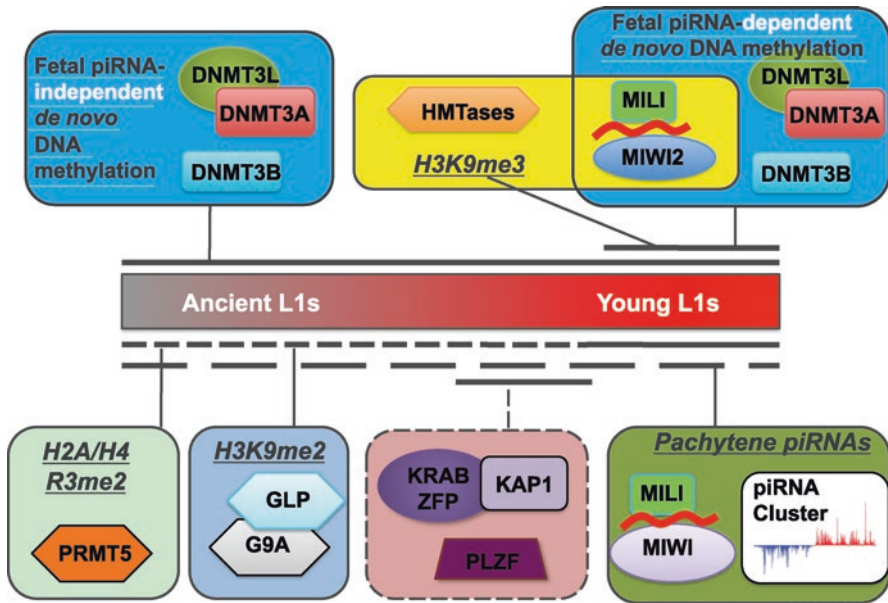


Fig. 3 L1 subfamily-specific regulation in the male germline. Next-generation sequencing-based approaches have revealed that L1 regulatory mechanisms differ in their subfamily specificity. MIWI2-guided de novo DNA methylation and H3K9me3 primarily target young active L1s through specificity granted by fetal piRNAs. In vitro data in ESCs suggest that KAP1, presumably guided by KRAB-ZFPs, recruits repressive histone marks to “middle-aged” L1s but not the youngest subfamilies. More general suppression occurs through piRNA-independent de novo methylation and posttranscriptional regulation by pachytene piRNAs. Pachytene piRNAs are generated from piRNA clusters and enrichment for specific subfamilies seems stochastic. Loss of H2A/H4R3me2 or H3K9me2 is associated with L1 activation in PGCs or spermatogonia. However, the distribution of either histone mark has not been defined for L1 subfamilies

perinuclear cytoplasmic granules that represent intramitochondrial cement. MIWI2 proteins, on the other hand, are both cytoplasmic and nuclear. In the cytoplasm, MIWI2 is localized to distinct granules sharing many components of the processing body. In the nucleus, MIWI2 distribution is homogenous. An early model built on observations from global *Mili* and *Miwi2* deletion mutants suggested that TE-derived fetal piRNAs are amplified through a heterotypic Ping-Pong loop, involving slicer activities of both MILI and MIWI2 (Aravin et al. 2008). However, when the slicer activity of MIWI2 is abolished by a point mutation, piRNA amplification is unaffected, both L1 and IAP remain repressed, and mutant males stay fertile (De Fazio et al. 2011). These results suggest that MIWI2 slicer activity is not required for piRNA amplification. In a revised model, MILI alone was proposed to drive the production of secondary piRNAs through an intra-MILI Ping-Pong cycle, which feeds anti-sense piRNAs to MIWI2 for subsequent targeted DNA methylation in the nucleus (De Fazio et al. 2011). Interestingly, in *Mili* slicer mutants, L1 is derepressed but

IAP remains silenced, indicating that L1 but not IAP silencing is dependent on piRNA amplification (De Fazio et al. 2011).

Transcriptional silencing by fetal piRNAs may also involve the repressive histone modification H3K9me3. SUV39H1/2 methyltransferases are known to specifically deposit H3K9me3 marks at pericentric and intergenic major satellite repeats. Using genome-wide ChIP-Seq approaches, two recent studies have provided novel insights into a functional role of H3K9me3 in transcriptional silencing of L1 retrotransposons (Bulut-Karslioglu et al. 2014; Pezic et al. 2014). In both somatic cells and premeiotic male germ cells (i.e., spermatogonia), H3K9me3 is enriched not only in major satellite repeats but also in L1s and LTR retrotransposons. However, its enrichment on L1s is much more pronounced in germ cells. Overall, transcriptionally active L1 sub-families (A, F, Gf, T), which contain the majority of full-length elements, display higher levels of H3K9me3. The distribution of H3K9me3 over full-length L1s is asymmetric, with significant concentration toward the 5'UTR promoter (Pezic et al. 2014). In spermatogonia from *Miw2*-deficient mice, levels of H3K9me3 are reduced from L1 A, Gf, and T elements, especially from the promoter region, but relatively constant at F elements. Coincidentally, L1 A, Gf, and T transcripts are elevated (Pezic et al. 2014). This study did not identify the histone methyltransferases responsible for H3K9me3 modification on L1 loci in the spermatogonia. In mouse ESCs, by comparing H3K9me3 distribution in wild-type and *Suv39h1/2*-deficient ESCs, L1s and ERVs are identified as the major targets of SUV39H1/2 outside constitutive heterochromatin. The 5'UTR of full-length L1s are specifically targeted for H3K9me3 modification. Accordingly, these L1s are derepressed in *Suv39h1/2*-deficient ESCs (Bulut-Karslioglu et al. 2014). Additionally, it has been previously shown that SUV39H1/2 are responsible for H3K9me3 in type B spermatogonia and preleptotene spermatocytes (Peters et al. 2001). *Suv39h1/2*-deficient male mice are infertile with most spermatocytes becoming apoptotic between mid- and late-pachytene stages (Peters et al. 2001). However, the effect of *Suv39h1/2* deletion on L1 expression in germ cells has not been reported. H3K9me3 also marks L1s and IAPs in E13.5 PGCs (Liu et al. 2014). However, conditional ablation of *Setdb1*, another H3K9 methyltransferase, in PGCs results in IAP upregulation only (Liu et al. 2014).

How fetal piRNAs target retrotransposons for de novo DNA methylation and/or H3K9me3 modification remains elusive. The observations that these marks are enriched at transcriptionally active L1 loci suggest a model in which fetal piRNAs guide the chromatin modifying apparatus to specific loci through interacting with nascent L1 transcripts (Molaro et al. 2014; Pezic et al. 2014). GTSF1 is a mouse protein with two tandem copies of the putative RNA-binding CHHC Zn-finger domain. Deletion of *Gtsf1* leads to phenotypes similar to those found in *Mili* or *Miw2* mutants (Yoshimura et al. 2009). Additional insights into the function of GTSF1 in the piRNA pathway came from flies. In *Drosophila*, Gtsf1 is required for Piwi-guided transcriptional silencing and deposition of H3K9me3 on retrotransposons, potentially mediating piRNA/target RNA interaction through its zinc finger domains (Donertas et al. 2013). Another *Drosophila* protein, CG3893 (Asterix), belongs to the same protein family characterized by the CHHC Zn-finger domain. Coincidentally, CG3893 is also essential for TE silencing and selected depo-

sition of H3K9me3 on certain transposons (Muerdter et al. 2013). Therefore, mouse GTSF1 may play roles analogous to Gtsf1 or Asterix in *Drosophila*, bridging the interaction between fetal piRNAs and nascent transcripts of targeted retrotransposons (Yu et al. 2015). It is unknown how piRNAs coordinate target-specific DNA methylation and histone modification. However, preliminary analysis of selected L1 loci indicates that H3K9me3 modification is uncoupled from DNA methylation (Pezic et al. 2014).

4.3 Transcriptional Silencing by Histone Modifications

Histone modifications have been well established as critical mechanisms in transcriptional silencing of ERVs in mouse ESCs and preimplantation embryos (Leung and Lorincz 2012), but little was known about their roles in L1 regulation during germ cell development until very recently. Compelling evidence indicates that two histone marks (H2A/H4R3me2 and H3K9me2; discussed below) repress L1 retrotransposons at different stages in the germline. In addition, as discussed in the previous section, H3K9me3 may function in fetal piRNA-mediated transcriptional silencing of L1s.

The symmetric methylation of arginine 3 of histones H2A and H4 (H2A/H4R3me2) is essential for transcriptional silencing of retrotransposons in PGCs (Kim et al. 2014). In PGCs, H2A/H4R3me2 is deposited by protein arginine methyltransferase 5 (PRMT5) (Ancelin et al. 2006). PRMT5 is expressed in both PGCs and somatic cells as early as E7.5 (Ancelin et al. 2006). It is transiently enriched in the nucleus of PGCs between E8.0 and E11.0, and later relocates back to the cytoplasm at E11.5 (Kim et al. 2014). The nuclear presence of PRMT5 is accompanied by progressive enrichment of H2A/H4R3me2 in PGCs from E8.5 to E10.5 and this modification persists at least until E12.5 (Kim et al. 2014) (Fig. 2). The function of H2A/H4R3me2 accumulation in PGCs was revealed by conditional ablation of *Prmt5* specifically in fetal germ cells (Kim et al. 2014; Li et al. 2015b). Both mutant males and females are sterile because of complete loss of PGCs by E16.5 (Kim et al. 2014; Li et al. 2015b). This early germ cell developmental defect is associated with an upregulation of L1 and IAP expression at both RNA and protein levels (Kim et al. 2014). In wild-type animals, both L1s and IAPs are enriched for H2A/H4R3me2 in PGCs at E10.5-E11.5. In mutant PGCs, H2A/H4R3me2 is globally lost with no significant changes in other chromatin markers, such as H3K9me2, H3K9me3, and H3K27me3. There is also no reduction of DNA methylation at L1 and IAP sequences. These results suggest PRMT5-mediated H2A/H4R3me2 modification is a dominant silencing mechanism for retrotransposons during PGC growth (Kim et al. 2014). This time period coincides with global DNA demethylation and loss of H3K9me2, when the germ cell genome is most vulnerable for retrotransposon activation, highlighting the importance of H2A/H4R3me2 modification in transcriptionally controlling retrotransposon expression in fetal gonads (Fig. 2).

The function of PRMT5 is not limited to its modification of histones. PRMT5 has many nonhistone protein substrates, including spliceosomal Sm proteins and Piwi proteins (Vagin et al. 2009; Kirino et al. 2009). Therefore, its function in PGC development may partially depend on its role in the methylation of Sm proteins. Indeed, conditional loss of *Prmt5* leads to aberrant splicing in PGCs (Li et al. 2015b). The PRMT5 protein also functions in the piRNA pathway by symmetrically dimethylating specific arginine residues of all three mouse Piwi proteins (Vagin et al. 2009). These modifications are required for proper interaction with specific Tudor family members (Vagin et al. 2009). In addition, PRMT5 is expressed postnatally during spermatogenesis. It is predominantly cytoplasmic in spermatogonia and nuclear in spermatocytes (Wang et al. 2015). Conditional deletion of *Prmt5* in postnatal male germ cells leads to loss of H2A/H4R3me2 and a meiotic defect. However, there is no significant change in L1 and IAP transcription (Wang et al. 2015). This observation does not necessarily refute a role in transcriptional silencing of retrotransposons by H2A/H4R3me2 modification in spermatocytes because other silencing mechanisms, such as DNA methylation, likely remain intact.

H3K9me2 marks silenced domains in euchromatin. It is catalyzed by the histone-lysine N-methyltransferase G9a/GLP heteromeric complex. The level of H3K9me2 is reduced in PGCs at E8.0 (Seki et al. 2005) and this low level is maintained in fetal prospermatogonia as a result of posttranscriptional downregulation of GLP (Deguchi et al. 2013). In postnatal mice, the H3K9me2 mark is detected in spermatogonia through the zygotene stage of spermatocytes (Fig. 2). The eventual loss of H3K9me2 is due to diminished G9a expression from the preleptotene stage (Di Giacomo et al. 2013, 2014). Conditional ablation of *G9a* in germ cells leads to the loss of H3K9me2 signal in postnatal germ cells, resulting in meiotic arrest at early pachytene stage, but has no impact on L1 and IAP expression (Tachibana et al. 2007). This unperturbed expression is not an indication that H3K9me2 is not involved in controlling L1 and IAP transcription. Instead it reflects remarkable functional redundancy among multiple retrotransposon silencing pathways (Di Giacomo et al. 2013). This multilayered regulation was revealed by comparing retrotransposon expression in different genetic backgrounds (Di Giacomo et al. 2014). Like many other piRNA pathway mutants, in *Mili*-deficient mice, L1s are derepressed only in spermatocytes but not in spermatogonia despite impaired piRNA biogenesis and the consequent loss of DNA methylation at L1 promoters. However, conditional inactivation of *G9a* in *Mili*-deficient adult mice aggravates the disruption of spermatogenesis: spermatogonia are the only germ cells remaining in seminiferous tubules of such animals (Di Giacomo et al. 2014). Unlike mice deficient in either *G9a* or *Mili* alone, L1 expression is derepressed in spermatogonia of double-deficient mice, indicating H3K9me2 is required to co-suppress L1 expression in spermatogonia (Di Giacomo et al. 2014). However, H3K9me2 alone is not sufficient in silencing IAP in spermatogonia, as evidenced by IAP upregulation in mice deficient for *Mili* alone (Di Giacomo et al. 2014). Thus, the programmed loss of H3K9me2 repression is the most likely cause of sudden TE expression at the onset of meiosis seen in many of the DNA methylation/piRNA pathway knockouts.

4.4 *Transcriptional Silencing Mediated by Zinc Finger Proteins*

Both human and mouse genomes encode a highly diverse family of transcription factors that can recognize specific target sequences through the tandem zinc finger DNA binding domains (Collins et al. 2001). The most abundant subgroup is known collectively as KRAB-zinc finger proteins (KRAB-ZFPs), which are characterized by the repressive Kruppel-associated box (KRAB) domain. A smaller subgroup comprises POZ-ZFPs, which is defined by the poxvirus and zinc finger (POZ) domain. Although a role in the germline has not been established, several members of KRAB-ZFP and POZ-ZFP families have been shown to mediate sequence-specific targeting and transcriptional silencing of L1 elements in other cellular contexts (Castro-Diaz et al. 2014; Jacobs et al. 2014; Puszyk et al. 2013).

KRAB-ZFPs are able to tether KRAB-associated protein 1 (KAP1, also known as tripartite motif-containing 28 or TRIM28) to the target site, which further recruits chromatin modifying enzymes to transcriptionally repress the target genes (Wolf et al. 2015). First demonstrated for exogenous retroviruses, KRAB-ZFPs also repress endogenous retrotransposons in both human and mouse ESCs. The depletion of KAP1 results in upregulation of specific subfamilies of LTR retrotransposons (e.g., IAP) (Rowe et al. 2010; Turelli et al. 2014) and L1s (Castro-Diaz et al. 2014). Interestingly, only “middle-aged” L1 subfamilies (e.g., full-length human L1PA5 and L1PA4 and mouse L1MdF2 elements) are subjected to KAP1-mediated suppression. Evolutionarily older and younger L1s are not affected in KAP1-depleted ESCs (Castro-Diaz et al. 2014) (Fig. 3). This phenomenon is consistent with evolutionary dynamics of KRAB-ZFPs and their retrotransposon targets. Simply put, a substantial amount of time is required for the host to evolve specific KRAB-ZFPs targeting active L1 lineages. Meanwhile, older L1s have accumulated sufficient mutations to obscure the original interaction with cognate KRAB-ZFPs. KRAB-ZFPs that are responsible for sequence-specific L1 targeting have started to emerge. Mouse L1MdF2 elements are specifically bound by Gm6871, a mouse-specific KRAB-ZFP, and derepressed in Gm6871-depleted mouse ESCs (Castro-Diaz et al. 2014). Primate-specific ZNF93 recognizes L1PA3-6 in the human genome (Jacobs et al. 2014). Most extraordinarily, a subgroup of L1PA3 elements, as well as the evolutionarily younger L1PA2 and L1PA1 subfamilies, are able to evade ZNF93-mediated transcriptional silencing due to a deletion at the ZNF93 binding site (Jacobs et al. 2014), highlighting an evolutionary arms race between retrotransposons and host factors.

Promyelocytic leukemia zinc finger (PLZF), encoded by *Zfp145*, is a member of the POZ-ZFP family (Collins et al. 2001). Acting as a transcription repressor, PLZF is an important factor in regulating cell growth and differentiation, typically acting through binding to specific DNA motifs and instigating repressive chromatin state locally by recruiting DNMTs and histone deacetylases. In the male germline, PLZF is first seen in prospermatogonia at E17.5 and subsequently restricted to undifferentiated spermatogonia after birth. Functional ablation of *Plzf* causes progressive loss

of spermatogonial stem cells and male infertility (Costoya et al. 2004). Interestingly, in a mouse model that is defective in PLZF DNA binding, methylated DNA immunoprecipitation (MeDIP) identified many hypomethylated L1 loci in the bone marrow and testis, and RT-PCR detected increased L1 RNA levels in these tissues (Puszyk et al. 2013). Follow-up experiments in cultured cells confirmed that many L1s contain a PLZF binding site in a conserved region of ORF2. PLZF binding appeared to establish and propagate DNA methylation and repressive histone marks to the 5'UTR of a full-length L1 element. Lastly, L1 expression was negatively correlated with PLZF rich tissues and those tissues had a closed chromatin conformation around the L1 sequences (Puszyk et al. 2013).

5 Posttranscriptional Silencing of L1s During Male Germ Cell Development

5.1 *Posttranscriptional Silencing by the Fetal piRNA Pathway*

As discussed earlier, much emphasis has been placed on the role of fetal piRNA in transcriptional silencing through targeting retrotransposons for DNA methylation. Its function in posttranscriptional silencing should not be ignored. Obviously, one way the fetal piRNA pathway can act posttranscriptionally is by cleaving TE transcripts into short piRNAs, which reduces the abundance of full-length TE transcripts in this process. The challenge is that most fetal piRNA pathway mutations have a deficit in de novo methylation of retrotransposons. So it is almost impossible to differentiate transcriptional from posttranscriptional mechanisms in these mutants. However, there are at least two cases where piRNA genesis was disrupted without a disruption of de novo methylation, revealing posttranscriptional silencing of TEs by the fetal piRNA pathway (Aravin et al. 2009; Ichihanagi et al. 2014).

Maelstrom (MAEL) contributes to posttranscriptional silencing of L1/IAP in fetal prospermatogonia (Aravin et al. 2009). Dynamics of L1 ORF1p expression has been examined in wild-type and *Mael* mutant germ cells from E14.5 to P10. In wild-type germ cells, L1 expression peaks at E16.5, when the L1 promoter has not been fully remethylated (61 % of CpGs methylated), but significantly declined by E18.5 and became almost undetectable by P2 (Aravin et al. 2009). This expression pattern is consistent with DNA methylation dynamics as L1 promoters are mostly remethylated by E18.5 (Fig. 2). In *Mael* mutant mice, L1 expression is similar to wild type at E16.5, but is further elevated in E18.5 germ cells. The high levels of ORF1p signals persist beyond P6. The increase in ORF1p is not due to loss of methylation as methylation levels are comparable to the wild type at E18.5 and P2. These results suggest that MAEL participates in posttranscriptional silencing of retrotransposons in fetal germ cells. The authors also reported that a similar mechanism targets IAPs (Aravin et al. 2009).

HSP90AA1 is one of the two isoforms of heat-shock protein 90 in mammals. HSP90AA1 protein is specifically expressed in germ cells of the mouse testis (Gruppi and Wolgemuth 1993). Deletion of *Hsp90aa1* causes a threefold reduction of fetal piRNA in E16.5 testes (Ichiyanagi et al. 2014). The levels of L1 ORF1p are increased in both E16.5 and E18.5 germ cells. However, L1 mRNA levels remain largely unchanged in E16.5 and P10. There is little change in L1 promoter methylation in P0 prospermatogonia between wild-type and mutant mice across multiple subfamilies, suggesting the presence of posttranscriptional regulation (Ichiyanagi et al. 2014).

5.2 *Pachytene piRNA Pathway*

Pachytene piRNAs were the first piRNA species reported in mammals (Aravin et al. 2006; Grivna et al. 2006a; Girard et al. 2006; Lau et al. 2006). Two mouse PIWI proteins, MIWI and MILI, are involved in pachytene piRNA biogenesis. MIWI expression is specific to meiotic and post-meiotic male germ cells (Deng and Lin 2002). It begins in mid-pachytene spermatocytes, peaks in diplotene spermatocytes, and persists in steps 1–3 round spermatids (Deng and Lin 2002). In adult animals, pachytene piRNAs are close to background level in spermatogonia, expressed at the highest level in spermatocytes, modestly expressed in round spermatids, but absent in elongating spermatids (Beyret and Lin 2011) (Fig. 2). In spermatocytes, MIWI is cytoplasmic; in spermatids, MIWI is cytoplasmic but enriched in chromatoid body (Grivna et al. 2006b). Deletion of *Miwi* causes spermatogenic arrest at step 4 in round spermatids (Deng and Lin 2002). The spermatogenic phenotype is accompanied by increased L1 RNA and ORF1p in purified round spermatids as well as in spermatocytes by immunofluorescence analysis (Reuter et al. 2011).

MIWI silences L1 retrotransposons posttranscriptionally. This was demonstrated by using a *Miwi* mutant that carries a point mutation in its conserved slicer domain DDH (termed *MiwiADH*) (Reuter et al. 2011). Similar to global *Miwi* knockout, spermatogenesis is arrested at the round spermatid stage in *MiwiADH* mutants. Unlike *Miwi* knockout, which does not express MIWI, *MiwiADH* mutant shows no change in MIWI protein abundance, subcellular localization, piRNA abundance, or genomic annotation. There is also no change in DNA methylation at L1 promoters in both *Miwi* and *MiwiADH* mutants. Nevertheless, L1 RNA and ORF1p are upregulated in both mutants. Together, these results suggest that MIWI silences L1s posttranscriptionally by cleaving L1 mRNAs (Reuter et al. 2011).

MILI's role in pachytene piRNAs cannot be interrogated by a global deletion of *Mili* because MILI is also involved in fetal piRNA biogenesis (see Sect. 4.2). Conditional deletion of *Mili* or its slicer activity (i.e., via a point mutation in the slicer domain) in spermatocytes, by crossing to Stra8-Cre mice, leads to a spermatogenic arrest at stages ranging from pachytene spermatocytes to elongating spermatids (Di Giacomo et al. 2013). Interestingly, L1, but not IAP, is upregulated only in late zygotene and pachytene spermatocytes, but not in surviving round and elongating

spermatids. Detailed analysis of MILI expression pattern reveals that, contrary to a common belief, which holds that MILI is continuously expressed in all stages of spermatogenesis, *Mili* ceases its expression from preleptotene to late zygotene stage (Di Giacomo et al. 2013) (Fig. 2). Because L1 methylation is unaffected in these mutant animals, these results suggest that MILI is required for posttranscriptional silencing of L1 at the onset of the zygotene/pachytene transition and in pachytene spermatocytes (Di Giacomo et al. 2013). MIWI expression and piRNA loading remain intact in these mutants, which could explain the lack of L1 derepression in spermatids.

Whether or not pachytene piRNAs themselves are necessary for L1 suppression is still unclear. MOV10L1 is an RNA helicase (Vourekas et al. 2015) that is required for both fetal (Frost et al. 2010; Zheng et al. 2010) and pachytene piRNA biogenesis (Zheng and Wang 2012). A conditional knockout of *Mov10l1* in spermatocytes displays limited pachytene piRNAs with no effect on fetal piRNA production. However, no derepression of L1 and IAP is observed in these mutants. These results suggest that pachytene piRNAs per se are not required for retrotransposon silencing (Zheng and Wang 2012). In these mutant mice, L1 transcripts may have been degraded by the slicer activity of MIWI through a pachytene piRNA-independent mechanism (Zheng and Wang 2012).

6 L1 Regulation During Female Germ Cell Development

The timing of mouse female germ cell development is significantly different from the male counterpart (Pepling 2006). The difference manifests shortly after PGCs have colonized the gonadal ridge at E10.5. Unlike male germ cells, which enter mitotic arrest between E12.5 and E14.5, the majority of female germ cells begin meiosis at E13.5–E15.5, becoming primary oocytes (Peters 1970; Baltus et al. 2006) (see also chapter “Retrotransposons and the Mammalian Germline” in this book). Primary oocytes progress through leptotene, zygotene, and pachytene stages, and become arrested at the diplotene stage of meiosis I between E17.5 and P5 (Peters 1970). Shortly after birth, oocytes become individually surrounded by somatic granulosa cells, forming primordial follicles (Peters 1969; Pepling and Spradling 2001). The majority of the primordial follicles will remain dormant for months or years until being recruited to the growing follicle pool. After the initial recruitment, primordial follicles must progress through primary, secondary, and antral stages before oocytes resume meiosis and ovulate as secondary oocytes resting at meiotic metaphase II (McGee and Hsueh 2000).

Male and female germ cells also differ in genome-wide DNA methylation dynamics. Unlike male germ cells, which have regained high levels of DNA methylation by E17.5, demethylation at the L1 promoter is protracted in fetal oocytes. There is no apparent remethylation even at E17.5 (Lees-Murdock et al. 2003; Seisenberger et al. 2012). In postnatal mice, primary oocytes arrested at the diplotene stage still display minimal methylation at L1 sequences (Sanford et al. 1987;

Howlett and Reik 1991). Methylation increases during meiosis, but L1 sequences remain modestly methylated in ovulated secondary oocytes (Howlett and Reik 1991), including active L1 A, Gf, and T subfamilies as evidenced by recent methylation studies (Smith et al. 2012).

Consistent with relaxed DNA methylation, L1 expression is readily detected in primary oocytes during the meiotic I prophase. L1 ORF1p is first detected at E15.5 in primary follicles and throughout all stages of prophase I in fetal and neonatal ovaries (Trelogan and Martin 1995; Malki et al. 2014). ORF1p signals were not found in adult ovaries (Trelogan and Martin 1995). Interestingly, ORF1p signals in the nucleus are highly variable among primary oocytes (Malki et al. 2014). Detailed analyses of nuclear ORF1p signals in E15.5, E18.5, and P5 oocytes suggest that differential L1 expression may play a role in fetal oocyte attrition, a programmed loss of developing oocytes in humans and rodents (Malki et al. 2014).

Among the three mouse Piwi genes, only *Mili* is expressed during female germ cell development (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2001; Aravin et al. 2008; Watanabe et al. 2008). MILI protein can be detected as early as E12.5 (Unhavaithaya et al. 2009; Aravin et al. 2008). The signal increases at E13.5 with the onset of meiosis but is significantly diminished at E18.5 (Unhavaithaya et al. 2009). Using transgenic animals that express GFP-tagged MILI under the endogenous *Mili* promoter, distinct MILI signals are detected in postnatal primary follicles and localized to cytoplasmic granules in both diplotene-arrested and growing primary oocytes (Aravin et al. 2008). Protein quantification indicates that MILI is more abundant in earlier stages of growing oocytes (Watanabe et al. 2008).

Although MILI is the only mouse Piwi gene expressed in female germ cells, piRNAs are produced in growing oocytes (Watanabe et al. 2008; Tam et al. 2008). The majority of these piRNAs are 25–26 nucleotides long and bound to MILI (Watanabe et al. 2008). Similar to fetal piRNA in prospermatogonia, oocyte piRNAs are enriched for retrotransposon sequences, including L1s and IAPs (Watanabe et al. 2008; Tam et al. 2008). Deletion of *Mili* leads to increased expression of L1s as well as IAPs (Watanabe et al. 2008; Lim et al. 2013). In addition to piRNAs, growing oocytes produce 21–22 nucleotides long small interfering RNAs (siRNAs) (Watanabe et al. 2006; Watanabe et al. 2008; Tam et al. 2008). Most of such siRNAs are derived from retrotransposons. In *Dicer*-deficient oocytes, neither IAP nor L1 transcription increases although other LTR retrotransposons are upregulated (Watanabe et al. 2008). Therefore, piRNAs and siRNAs complement each other to regulate distinct retrotransposon families in growing oocytes.

7 Other Factors Implicated in L1 Regulation

The male germline is a potential hub for retrotransposition. A recent study suggests that, for all three active classes of retrotransposons (L1, SINE, and LTR), new insertions occur almost exclusively through the male germline genome (Nellaker et al. 2012). Using whole-genome sequencing data, the authors catalogued polymorphic TE

variants among 18 inbred mouse strains (Nellaker et al. 2012). Based on the evolutionary divergence among the mouse strains analyzed, these polymorphic TE variants represent “recent” insertion events within the past two million years. Interestingly, when chromosomal distribution was analyzed, all classes of polymorphic variants (L1, SINE, and LTR) were relatively depleted on the X chromosome. The deficit of polymorphic TEs on the X chromosome contrasts sharply with fixed TEs, which are distributed evenly among all chromosomes (an exception is fixed L1s, which are enriched on the X chromosome due to positive selection). The relative depletion of TE variants on the X chromosome versus autosomes is indicative of a male-driven insertional mechanism. Simply put, if retrotransposition occurs at equivalent rates in males and females, there should be no chromosomal bias. However, the calculated ratio of male-to-female insertion rates is 7.3, 151.8, and 7.8 for polymorphic L1, SINE, and LTR variants (Nellaker et al. 2012).

What might be responsible for this apparent gender bias in retrotransposition? A prime candidate is the vast difference in the number of germ cell divisions between male and females (Drost and Lee 1995). Mouse PGCs have committed to sex-specific trajectories by E13.5. From this point on, female germ cells enter meiosis and meiotically divide twice to become eggs, whereas male germ cells undergo a minimum of 9 mitotic divisions plus two meiotic divisions (i.e., 11 divisions) to become sperm at the end of the first wave of spermatogenesis (de Rooij and Russell 2000). The number of cell divisions can be substantially higher in adult animals as spermatogonia stem cells divide every 8.5 days to sustain continued spermatogenesis (Oakberg 1957). For example, the corresponding number of cell divisions would be around 17 for a 3-month-old male mouse. Thus, the ratio of male-to-female cell divisions would be ~8.5 (e.g., 17 divided by 2). This back-of-the-envelope calculation suggests that the bulk of retrotransposition in the male germline must occur before male meiosis, either in prospermatogonia or dividing spermatogonia.

Mitotically dividing spermatogonia might be the most important cellular niche for retrotransposition. In vitro experiments demonstrate that cell division promotes L1 retrotransposition (Shi et al. 2007; Xie et al. 2013). In these experiments, retrotransposition of engineered L1 constructs is diminished in cell-cycle arrested cells (Shi et al. 2007; Xie et al. 2013) and non-dividing cells (Kubo et al. 2006). Even in dividing but cell-cycle synchronized cells, retrotransposition frequency is lower if cells just go through one fewer cell division (Xie et al. 2013). These results suggest that the intact nuclear membrane may act as a barrier for L1 RNP to access the nuclear genome (Fig. 1). A recent analysis of polymorphic processed pseudogenes in the 1000 Human Genomes Project supports the importance of cell division in L1-mediated retrotransposition (Abyzov et al. 2013). Processed pseudogenes are copies of cellular mRNAs that have been reversed transcribed and inserted into the genome by the L1 machinery (Esnault et al. 2000). The first piece of evidence is that polymorphic processed pseudogenes are enriched for parent genes being expressed during M or M/G1 transition. The second piece of evidence is that M/G1 expressed genes possess higher copies of fixed processed pseudogenes. Both are consistent with a model in which retrotransposition is coupled to cell division (Abyzov et al. 2013). The potential requirement for active cell division may help to explain a peculiar

observation from L1 transgenic mice (Kano et al. 2009). Despite abundant RNA signals in male meiotic and post-meiotic germ cells, no heritable L1 insertions were detected in these L1 transgenic mice. Part of this “posttranscriptional block” to retrotransposition may be the expectedly low number of cell divisions by spermatocytes (e.g., twice for primary spermatocytes, once for secondary spermatocytes, and no further division for spermatids and sperm), although the unique properties of meiotic chromatin and spermatid DNA compaction may present a formidable challenge to the retrotransposition machinery (Fig. 1). However, it has not been formally shown that a cell cycle arrest *in vivo* blocks L1 retrotransposition.

8 L1 Expression and Regulation in Human Germ Cells

The vast majority of the findings discussed above were obtained from mouse models. How do they translate into humans? Due to tissue accessibility issues, time points of L1 expression in human germ cells are rather limited but the overall expression pattern is similar to what has been found in mice (Rosser and An 2012). Both human L1 ORF1p and ORF2p are detectable in fetal and adult testes (Ergun et al. 2004). L1 protein expression has not been reported in human ovaries. As for DNA methylation, multiple recent methylomic analyses indicate that global demethylation is conserved in human PGCs (von Meyenn and Reik 2015; Guo et al. 2015; Tang et al. 2015; Gkoutela et al. 2015). However, global demethylation is not accompanied by significant derepression of retrotransposon transcripts in human PGCs, suggesting other silencing mechanisms are in place. H3K9me3 has been suggested as a candidate. It is consistently present during this stage of germ cell development (Gkoutela et al. 2015; Guo et al. 2015; Tang et al. 2015), but whether H3K9me3 is enriched on retrotransposons has not been investigated.

Human germ cells also have an intact piRNA pathway. The human genome encodes four Piwi proteins: HILI (MILI for mouse), HIWI1 (MIWI for mouse), HIWI2 (MIWI2 for mouse), and HIWI3 (Sasaki et al. 2003). At the RNA level, HILI and HIWI are expressed specifically in testes and ovaries (Sasaki et al. 2003; Williams et al. 2015). HILI and HIWI proteins have also been confirmed in adult ovaries (Roovers et al. 2015). Unlike mouse MIWI2, which is restricted to embryonic/neonatal male germ cells, HIWI2 is ubiquitously expressed in normal human tissues (Keam et al. 2014; Williams et al. 2015). HIWI3, which has no ortholog in mouse, is also detected specifically in human testes and ovaries, but its transcript abundance is at least tenfold lower than its three other paralogs (Williams et al. 2015). Pachytene piRNAs are abundant in adult human testes (Aravin et al. 2006; Girard et al. 2006; Ha et al. 2014; Yang et al. 2013; Williams et al. 2015). They can be enriched by antibodies against HILI and HIWI, suggesting these piRNAs are HILI and HIWI-bound (Williams et al. 2015). piRNAs are also present in human ovaries, and more abundant in fetal ovaries than in adult ovaries (Williams et al. 2015; Roovers et al. 2015). The role of the piRNA pathway on human L1 regulation has not been determined. There is a general lack of enrichment of retrotransposon

sequences in piRNA libraries isolated from human adult testes and fetal ovaries (Williams et al. 2015; Roovers et al. 2015). However, in parallel to male infertility found in *Miw12*-deficient mice, increased L1 ORF1p expression is correlated with reduced HIWI2 expression in the spermatogonia of boys with cryptorchidism who are at a high risk of infertility (Hadziselimovic et al. 2015).

9 Functional Consequences of L1 (De)regulation in the Germline

9.1 Implication on the Timing of L1 Retrotransposition

Both the frequency and timing of new L1 insertions remain unexplored during germline development. Nevertheless, recent studies have improved and refined our knowledge about L1 expression during normal germ cell development (Sect. 3). L1 is expressed at both RNA and protein levels in both male and female germ cells. For males, there are two developmental time windows (Fig. 2); for females, only in meiotic germ cells. How do these restricted expression windows impact L1 retrotransposition? As transcriptional activation is a prerequisite for L1 retrotransposition, it is expected that the bulk of retrotransposition in germ cells occurs during these time windows. As discussed in Sect. 7, retrotransposition may also favor actively dividing cellular stages. One caveat is that, for the great majority of studies, ORF1p is used as a surrogate for L1 expression at the protein level. This is because endogenous ORF2p has been notoriously difficult to detect, partially due to its low expression levels (Dai et al. 2014). As ORF2p function is required for L1 retrotransposition (Moran et al. 1996), the control of ORF2p expression may be a key mechanism. L1 ORF2p has been detected in human fetal and adult testes (Ergun et al. 2004), but a careful analysis of ORF2p during germ cell development is lacking in mouse models.

The timing of retrotransposition in the male germ line will have a huge impact on the allelic frequency of new L1 insertions in the resulting spermatozoa. If an insertion occurs at the prospermatogonia/spermatogonia stage, it may be represented in thousands of gametes. In contrast, if retrotransposition occurs at the spermatid stage, this specific insertion can only be found in a single gamete. Thus, early retrotransposition timing increases the allelic frequency for a specific insertion by several thousand-fold as compared to late retrotransposition events. The above interpretation is based on the premise that all insertions are selectively neutral and do not affect the fitness of afflicted germ cells. However, the orientation bias of intronic L1 and LTR elements in the human genome suggests that some TE insertions are subjected to purifying selection (Smit 1999; Medstrand et al. 2002). Even if a small fraction of the insertions are deleterious or adaptive mutations, the difference in retrotransposition timing will significantly impact the overall fitness landscape. This is because late insertions, by definition, occur only in terminal stage of germ cell development and the selection pressure will act on a limited number of developmental pathways (i.e., post-meiotic development for spermatids). In contrast, early-stage germ cells carry-

ing insertions have to survive a myriad of additional developmental processes and eventually emerge as *de novo* mutations in the population (i.e., the journey for prospermatogonia to become spermatozoa). A corollary of germ cell selection is that the actual frequency of retrotransposition in developing germ cells can be substantially higher than what would be observed in live births. Future studies in animal models are required to address these important questions.

9.2 *Impact on Germ Cell Development*

What is the functional consequence of retrotransposon deregulation? As discussed above, genetic ablation of various host factors leads to retrotransposon derepression, evidenced by increased RNA and protein expression. Indeed, some of these mechanisms preferentially target transcriptionally and transpositionally active retrotransposon lineages (Fig. 3). In mice, these include several evolutionarily young non-LTR and LTR-retrotransposon subfamilies, such as L1s and IAPs, which can potentially wreak havoc to the germline genome due to insertional mutagenesis. A common theme emerging from these mouse knockout studies is a disruption of the spermatogenic program and subsequent male infertility. An unanswered question is whether spermatogenic failure is caused by the derepression of retrotransposons itself. One of the biggest obstacles is how to quantitatively determine the number of new insertions while facing an extremely high baseline signal from preexisting retrotransposon copies. Real-time PCR has been used to quantify endogenous human and mouse L1 copies (Coufal et al. 2009; Muotri et al. 2010). A similar strategy failed to detect any significant changes in L1 copy number in *Dnmt3l*-deficient mice (Zamudio et al. 2015). Therefore, there is an urgent need for novel, sensitive detection methods so that the relationship between L1 expression and mobilization *in vivo* can be established. It is also important to emphasize that TE activation can potentially act on germ cells at three different stages of the replication cycle: transcription, translation, and insertion (Fig. 1). Besides insertional mutagenesis, overexpression of L1 ORF2p is known to be cytotoxic (Belgnaoui et al. 2006; Gasior et al. 2006; Malki et al. 2014; Wallace et al. 2008). Additionally, transcriptional activation of normally silenced L1 loci genome-wide has the potential to interfere with chromosome pairing, synapses, and recombination (Zamudio et al. 2015).

10 **Concluding Remarks**

It has been a decade since the initial discovery of mammalian piRNAs in 2006. Follow-up studies spotlight critical roles of fetal and pachytene piRNA pathways for retrotransposon regulation in the male germline. Remarkable progress has also been made in delineating the intricate relationships between piRNAs, DNA methylation, and histone modifications. It is clear that the host has evolved multiple

layers of mechanisms to prevent uncontrolled retrotransposon activities at each stage of the male germ cell development (Fig. 2). Retrotransposons are demethylated in post-migratory PGCs, which rely on H2A/H4R3me2 modification to transcriptionally silence L1 and IAP (Sect. 4.3). The mitotically arrested prospermatogonia are protected by the fetal piRNA pathway, which establishes DNA methylation (and potentially H3K9me3) by birth (Sect. 4.2). After birth, retrotransposons are silenced in spermatogonia by H3K9me2, in addition to DNA methylation (Sect. 4.3). In meiotic germ cells, retrotransposons are transcriptionally silenced by DNA methylation and posttranscriptionally suppressed by pachytene piRNAs (Sect. 5.2). In contrast, our understanding of how retrotransposons are regulated in the female germline is much less complete (Sect. 6). Even less is our knowledge about L1 regulation in human germ cells (Sect. 8). It is imperative to understand how different repression systems cooperate to suppress individual retrotransposons. Future studies can no longer view all L1s as equivalent and sub-families should be separately analyzed (Fig. 3). Furthermore, analysis must be germ cell specific and stage specific. Tools to quantify retrotransposition must be improved and new methods be developed.

Acknowledgments Research in our laboratory is supported by the National Institutes of Health (NIH) grants R21OD017965, R21HD080143, and P50GM107632. SN was supported, in part, by NIH award T32GM008336. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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Human Retrotransposons in Health and Disease

Cristofari, G. (Ed.)

2017, X, 330 p. 34 illus., 28 illus. in color., Hardcover

ISBN: 978-3-319-48343-6