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During the past several years, it has become clear that small RNAs guard germ cell genomes from the activity of mobile genetic elements. Indeed, in mammals, a class of small RNAs, known as Piwi-interacting RNAs (piRNAs), forms an innate immune system that discriminates transposons from endogenous genes and selectively silences the former. piRNAs enforce silencing by directing transposon DNA methylation during male germ cell development. As such, piRNAs represent perhaps the only currently known sequence-specific factor for deposition of methylcytosine in mammals. The three mammalian Piwi proteins Miwi2, Mili, and Miwi are required at different stages of germ cell development. Moreover, distinct classes of piRNAs are expressed in developmental waves, with particular generative loci and different sequence content distinguishing piRNAs populations in embryonic germ cells from those that appear during meiosis. Although our understanding of Piwi proteins and piRNA biology have deepened substantially during the last several years, major gaps still exist in our understanding of these enigmatic RNA species.

GENERAL MECHANISMS OF RNA SILENCING

RNA interference (RNAi) was originally discovered as an artificial process of silencing cellular mRNAs in response to the addition of exogenous double-stranded RNAs (Fire et al. 1998). The power of this response strongly hinted at an endogenous function for such mechanisms, and work in the field quickly revealed that multiple, RNAi-related pathways formed a previously unrecognized layer of gene regulation and genome defense that is essential to virtually all eukaryotic organisms. The development of methods to characterize small RNA sequences opened previously unseen worlds of genomic output in organisms ranging from plants and fungi to protozoans and mammals. These fell into a number of classes based on their biogenesis mechanisms, genomic origins, and biological functions. Thus far, small RNAs that act in RNAi-related pathways fall mainly into these three classes.

In a classical RNAi experiment, exogenously provided, long double-stranded RNA is processed in the cell by an RNase III–family nuclease, Dicer, into mature small interfering RNAs (siRNAs) of 21–23 nucleotides (Bernstein et al. 2001; Elbashir et al. 2001). One strand of siRNA selected and incorporated in the RNA-induced silencing complex (RISC), which contains at its core a member of the Argonaute protein family (Hammond et al. 2001; Martinez et al. 2002; Liu et al. 2004). This protein directly interacts with both the 5′ and 3′ ends of the small RNA and uses it as a guide to recognize complementary targets. In classical RNAi, Argonaute also performs silencing by cleaving target transcripts using an endogenous, RNase H–related nuclease domain (Liu et al. 2004; Meister et al. 2004; Song et al. 2004). Endogenous siRNAs have revealed themselves in plants, fungi, and animals, wherein they have roles in gene regulation, transposon control, and chromosome organization.

microRNAs (miRNAs) share many similarities with siRNAs. They are processed from largely double-stranded precursors, produced from the genome by transcription of short, imperfect inverted repeats (Bartel 2004). The defining difference between miRNAs and siRNAs in animals is the addition of a nuclear processing step: the conversion of the primary miRNA (pri-miRNA) transcript into the pre-miRNA by Drosha, another RNase III family enzyme (Lee et al. 2003; Denli et al. 2004; Han et al. 2004). Following export to the cytoplasm (Kim 2004), maturation into an siRNA-like species is accomplished by Dicer (Grishok et al. 2001; Hutvagner et al. 2001), and the mature miRNAs then joins an Argonaute protein (Hutvagner and Zamore 2002; Mourelatos et al. 2002). In this case, target recognition does not depend on the entire extent of the small RNA but instead on a short guide sequence comprising roughly nucleotides 2–8 of the miRNA (Lewis et al. 2003, 2005; Stark et al. 2003). Most often, this region binds complementary sites in the 3′ UTRs of its regulatory targets, impacting their expression by regulation of translation and stability. In plants, relationships between siRNAs and miRNAs are less clear, because plants have no Drosha equivalent and appear to have evolved miRNA-mediated regulation separately. Plants do appear to have specialized different Dicer proteins for the production of miRNAs and siRNAs (Xie et al. 2004); however, a full understanding of the biochemistry of these pathways is still emerging.

The third class of small RNAs appears to be specific to animals and interacts with an animal-specific clade of Argonaute proteins, the Piwi family (Aravin et al. 2007a). These Piwi-interacting RNAs (piRNAs) have been found in *Caenorhabditis elegans* (Batista et al. 2008; Das et al. 2008), *Drosophila* (Saito et al. 2006; Brennecke et al. 2007), zebra fish (Houwing et al. 2007, 2008), and mammals (Aravin et al. 2006, 2007b; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Watanabe et al. 2006), where their expression is most prominent in male and female germ cells. In most organisms, piRNAs have clear roles in guarding germ cell genomes from the activity of mobile genetic elements (Aravin et al. 2007b; Brennecke et al.

2007; Houwing et al. 2007). They accomplish this as part of an elegantly constructed innate immune system that recognizes and silences transposons through both transcriptional and posttranscriptional mechanisms. However, it is clear that piRNAs must also have additional biological roles that are essential to germ cell maturation.

Although the roles of small RNAs are ubiquitous, their importance is particularly high in animal stem and germ cells. In stem cells, entire classes of miRNAs appear to be excluded from expression, instead defining a variety of differentiated cell fates. In germ cells, the broadest variety of small RNAs is elaborated, underscoring the special requirement for these pathways in reproductive tissues.

MIRNA AND SIRNA PATHWAYS IN MAMMALIAN STEM AND GERM CELLS

Small RNA silencing pathways have important roles in controlling gene expression in many if not all of the cell types of multicellular organisms. Mice deficient in *Dicer*, a key enzyme required for miRNA and siRNA biogenesis, die before gastrulation (Bernstein et al. 2003), preventing analysis of Dicer function at later stages of development. Indeed, the ability of mice to sustain 7.5 days of development is probably due, at last in part, to the maternal contribution of Dicer and processed small RNAs (Tam et al. 2008; Watanabe et al. 2008). Production of conditional Dicer alleles by several groups indicated that small RNA pathways are required for the maintenance of embryonic stem (ES) cell potency and for the correct development of many different organ systems (Cobb et al. 2005; Harfe et al. 2005; Kanellopoulou et al. 2005; Murchison et al. 2005; Harris et al. 2006). Dicers have also proven essential for development in *Drosophila*, zebra fish, *Arabidopsis*, and many other systems in which mutant alleles have been analyzed.

Analysis of small RNA profiles in different tissue types revealed sets of ubiquitous as well as cell-type-specific miRNAs. For example, human and mouse ES cells express a group of miRNAs that are not present or exist only at very low levels in somatic cells (Houbaviy et al. 2003; Suh et al. 2004). One particular ES-cell-specific miRNA family, miR-290–295, was suggested to indirectly control the expression of de novo DNA methyltransferases by silencing the repressor protein Rbl2 (Benetti et al. 2008; Sinkkonen et al. 2008). A comprehensive investigation of small RNA profiles in adult stem cells is still underway, and it is currently not clear whether there is a common stem cell miRNA signature. However, a number of miRNAs that appear to be widely expressed in most differentiated cell types, such as the *let-7* family, are present at relatively low levels in tissue stem cells (Viswanathan et al. 2008). Overall patterns of miRNA expression have also been shown to track cell-fate specification in well-studied systems, such as hematopoietic development (Chen et al. 2004; Zhou et al. 2007).

Small RNA profiling in different tissues and cell types of mammals has revealed a plethora of miRNAs, with more than 800 species in humans that have substantial experimental support (Griffiths-Jones 2006; Landgraf et al. 2007). However, until recently such studies failed to reveal endogenous siRNAs (endo-siRNAs). The absence of abundant endo-siRNAs in mammals is likely explained by the activity of the interferon/protein kinase R (PKR) pathway that arrests translation in the presence of long double-stranded RNAs. Two groups of investigators, including our own, identified endogenous siRNAs in mouse oocytes, a cell type that demonstrably lacks a strong PKR pathway (Tam et al. 2008; Watanabe et al. 2008). One source of endogenous siRNAs is transposable elements (TEs); however, a small number of protein-coding genes also produce endo-siRNAs. In both cases, formation of double-stranded RNAs would require transcription of both strands of a particular locus. In the case of transposons, one can easily imagine that some copies might have integrated near a promoter that could produce antisense transcripts (but see below). However, the source of antisense information for protein-coding genes was mysterious. Deep sequencing and the analysis of polymorphic sense and antisense siRNAs corresponding to protein-coding genes allowed assignment of antisense information specifically to pseudogenes. Thus, the gene and pseudogene transcripts must form intermolecular hybrids that are substrates for Dicer. In part, the efficient interaction of transcripts from discrete genomic loci might hinge on the unique capacity of the oocyte to serve as a storehouse for RNAs that drive early development of the zygote.

The functional relevance of endo-siRNAs is supported by the observation that lesions in the RNAi pathway impact their target genes. For example, in Dicer-deficient oocytes, expression of endo-siRNA targets is increased (Tam et al. 2008; Watanabe et al. 2008). Genetic evidence also supports a role for Ago2, the only catalytically competent mammalian family member, in this regulatory circuit (Watanabe et al. 2008). Endo-siRNA targets are enriched for genes related to microtubule dynamics (Tam et al. 2008), and Dicer mutants show severe chromosome segregation and spindle defects during oocyte maturation (Murchison et al. 2007). This suggests that pseudogenes might not simply be molecular artifacts but might also have essential roles in gene regulation. There is also an intriguing possibility, fueled by recent studies of piRNAs in *Drosophila* (Brennecke et al. 2008), that inherited small RNA populations might have roles during early development.

Recently, endo-siRNAs have also been reported in ES cells (Babiarz et al. 2008), another context in which PKR-mediated nonspecific responses to dsRNA are not strong. In contrast, deep sequencing efforts have failed to reveal endo-siRNAs in a variety of somatic cell types. endo-siRNAs are seen in both somatic and germ cell compartments of *Drosophila* (Czech et al. 2008; Kawamura et al. 2008; Okamura et al. 2008) and *C. elegans* (Ambros et al. 2003), suggesting that there is no conserved restriction of these species to germ or multipotent cell types.

FUNCTION OF PIWI PROTEINS IN GERM CELLS

The third class of small silencing RNAs, piRNAs, and their protein partners, the Piwis, have expression patterns

that are largely restricted to germ cells. In *Drosophila*, which has three members of the Piwi subfamily, these proteins are expressed in male and female germ cells (Brennecke et al. 2007). One family member, Piwi itself, is additionally expressed in follicular cells that have somatic origin but that tightly associated with and form a niche for germ-line cells. In *C. elegans* (Cox et al. 1998; Batista et al. 2008), zebrafish (Houwing et al. 2007, 2008), and mouse (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004; Aravin et al. 2008), the expression of Piwi family members is also restricted to germ-line cells. Interestingly, in more primitive animals, such as flatworms, Piwis are expressed in pluripotent neoblast cells that are responsible for the amazing regeneration potential of these animals and that can differentiate in any type of somatic or germ-line cell (Reddien et al. 2005).

The intracellular localization of Piwis also implicates them in germ-cell-specific processes. Most Piwi family members appear in cytoplasmic granular structures called nuage/germ plasm (*Drosophila*) or P granules (*C. elegans*) that mark germ cells throughout their development (Brennecke et al. 2007; Houwing et al. 2007; Aravin et al. 2008; Batista et al. 2008). Germ plasm/P granules have been strongly linked to germ cell-fate determination in multiple organisms. Interestingly, nuage-like granules containing Piwi proteins are present in both germ cells and neoblast cells in flatworms (Palakodeti et al. 2008). The localization of Piwi proteins seems linked to their function, and it likely that one of the major roles of nuage/germ plasm is related to their relationship to small RNA pathways. However, these structures also contain proteins that have not yet been linked to small RNA biology, suggesting that these locales likely have additional roles in RNA metabolism.

In mouse, determination of germ cells occurs relatively late in development and inheritance of nuage granules seems to have no role in this process. However, granules containing one of the Piwi proteins, MILI, are present in female germ cells from the arrested to the growing oocyte stage (Aravin et al. 2008). In embryos, the presence of MILI granules can be detected in both sexes soon after migrating primordial germ cells reach the somatic gonadal ridge (E12.5). Therefore, although Piwi-containing granules in mammals may not be directly involved in germ cell determination, even here they remain tightly associated with germ cells throughout almost all stages of gametogenesis.

Genetic analyses have also supported critical roles for Piwi proteins in germ cell development. Individual mutants for any of three Piwi family genes in mouse are male-sterile (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004, 2008; Carmell et al. 2007), whereas in zebra fish and *Drosophila* both male and female fertility is affected. No drastic defects in the development of somatic cells is observed upon Piwi mutation in any of these three organisms, although several reports indicate that Piwi family members in *Drosophila* might be involved in heterochromatin formation in somatic cells (Pal-Bhadra et al. 2004; Yin and Lin 2007). In flatworms, Piwi expression is critical for maintaining the regenerative potential of neoblasts (Reddien et al. 2005).

A detailed characterization of mutant phenotypes has revealed a number of specific developmental and molecular defects. In mouse, *Miwi*-mutant male germ cells arrest gametogenesis at the postmeiotic, round spermatid stage (Deng and Lin 2002). In *Mili* and *Miwi2* mutants, spermatogenic arrest occurs during meiosis with visible defects in chromosome alignment (Kuramochi-Miyagawa et al. 2004; Carmell et al. 2007). Deficiency in *Mili* and *Miwi2* also leads to defects in spermatogenic stem cell maintenance that manifests itself in a progressive loss of germ cells in adult animals (Carmell et al. 2007; Unhavaithaya et al. 2008). Analogously, mutation of Piwi family members in *Drosophila* causes defects at various stages of oogenesis. *Aub* mutants have defects in oocyte polarity and axis specification (Harris and Macdonald 2001). These defects are suppressed by mutations in *ATR* and *Chk2*, genes that encode checkpoint kinases that respond to nonrepaired double-stranded DNA breaks formed during meiosis (Klattenhoff et al. 2007). This result and the finding of unrepaired DNA breaks in *Miwi2*-deficient mouse cells (Carmell et al. 2007) suggest that the phenotype of at least some Piwi mutations in *Drosophila* and mouse might be secondary consequences of activating the DNA-damage checkpoint. Mutation of the founding member of the Piwi subfamily, Piwi itself, in *Drosophila*, causes depletion of stem cells in the ovary (Cox et al. 1998, 2000), similar to *Mili*- and *Miwi2*-mutant phenotypes in mouse. However, in contrast to mouse, the *Drosophila* Piwi defect seems to be noncell autonomous; lack of Piwi protein in somatic follicular cells results in defects in germ-line stem cell maintenance. Therefore, at least partially, the role of Piwi in *Drosophila* might be to maintain a proper somatic niche for germ-line stem cells.

Although analysis of Piwi-deficient mutants demonstrated links to germ cell maintenance, meiosis, and DNA-damage checkpoints, understanding the molecular mechanisms of Piwi function was impossible without identifying the Piwi small RNA binding partners, piRNAs.

piRNA POPULATIONS IN GERM CELLS

piRNAs derived from repetitive elements were initially identified in total RNA isolated from *Drosophila* germ cells and were termed repeat-associated small interfering RNAs (rasiRNAs) (Aravin et al. 2003). Subsequent studies showed that rasiRNAs significantly differed from siRNA and deserved separation in specific class (Vagin et al. 2006). piRNAs arise by a Dicer-independent mechanism that results in mature species that are larger than miRNAs and siRNAs. The precursors to piRNAs do not have the pronounced double-stranded structure that is a signature of miRNAs and siRNAs. Genomic mapping of piRNAs showed that multiple piRNAs mapped within discrete genomic intervals, forming so-called piRNA clusters that can exceed 100 kb. Each piRNA cluster produces numerous piRNAs that can have overlapping sequences, generating an amazing diversity of mature piRNA species. Although the majority of piRNA clusters in *Drosophila* produce piRNAs that match to both

genomic strands, some piRNA clusters are single stranded (Brennecke et al. 2007). Similarly, piRNA clusters in mouse and zebra fish are either single stranded or consist of several segments in which the polarity of piRNA production switches between the plus and minus strands (Aravin et al. 2006, 2008; Girard et al. 2006; Houwing et al. 2007). Such a structure suggests that piRNAs are processed from long, single-stranded, precursor molecules that traverse the piRNA cluster. Indeed, insertional mutations in the putative promoter region of one of the *Drosophila* piRNA clusters, *flamenco*, eliminate piRNAs produced from the cluster as much as 100 kb away from the site of insertion (Brennecke et al. 2007).

Deep sequencing of piRNA libraries cloned from total cellular RNAs or immunoprecipitated Piwi complexes from *Drosophila*, *C. elegans*, zebra fish, and mammals revealed two types of piRNAs. Nonrepetitive piRNAs start to become expressed during the pachytene stage of meiosis in mouse germ cells and were accordingly termed pachytene piRNAs (Aravin et al. 2006; Girard et al. 2006). Similarly, piRNAs in *C. elegans*, called 21U RNAs, are derived from unique genomic regions and match only to the sites in the genome from which they are derived (Batista et al. 2008; Das et al. 2008). In contrast, a majority of piRNAs in *Drosophila* and zebra fish correspond to repetitive genomic elements, particularly different TEs. Whereas *Drosophila*, *C. elegans*, and zebra fish seem to have only one type of piRNA (repetitive or nonrepetitive), germ cells in mouse express both types at different stages of spermatogenesis (Aravin et al. 2007b, 2008; Kuramochi-Miyagawa et al. 2008). Expression of nonrepetitive, pachytene piRNAs is preceded by a population of piRNA derived from repeats. These initiate expression in germ cells during embryogenesis. Furthermore, expression of repetitive piRNAs during spermatogenesis is dynamic and regulated in a developmental fashion.

Nonrepetitive, pachytene piRNAs appear only at meiosis and interact with MIWI and MILI proteins (Aravin et al. 2006; Girard et al. 2006). Loss of these species is likely responsible for postmeiotic arrest of spermatogenesis observed in *Miwi*-deficient animals. Currently, the molecular function of pachytene piRNAs in mouse and of 21U piRNAs in *C. elegans* remains a matter of speculation, but it likely involves a germ-cell-specific process that occurs during and/or after meiosis.

The repetitive nature of the other class of piRNAs immediately suggested their function in the silencing of repetitive genomic elements, particularly transposons. This function is strongly supported by mutational analysis of Piwi proteins. In *Drosophila*, zebra fish, and mouse, deficiency for Piwi family members leads to overexpression of several types of repetitive elements in germ cells (Sarot et al. 2004; Kalmykova et al. 2005; Vagin et al. 2006; Aravin et al. 2007b; Carmell et al. 2007; Houwing et al. 2007). piRNA-producing loci have not yet been subjected to systematic genetic analysis, but mutations that lead to the perturbed function of one locus, *flamenco/COM*, were identified in *Drosophila* (Prud'homme et al. 1995; Desset et al. 2003). The *flamenco/COM* locus controls the expression of at least three different retrotransposons—*gypsy*, *Zam*, and *idefix*—and mutations that activate transposon expression also eliminate piRNA production from the locus (Brennecke et al. 2007).

Activation of TEs is likely responsible for the phenotypes of Piwi mutants in *Drosophila* and zebra fish and of *Mili* and *Miwi2* (but not Miwi) knockouts in mouse. It was proposed that active transposition in these mutant animals generates double-stranded breaks that are detected by the ATR/chk2 checkpoint and that, in turn, lead to meiotic arrest (Klattenhoff et al. 2007). Indeed, numerous Spo11-independent DNA breaks were detected in Piwi mutants in *Drosophila* (Klattenhoff et al. 2007) and mouse (Carmell et al. 2007).

It is less obvious how activation of TEs might affect the maintenance of germ-line stem cells, which are defective in *Piwi* mutants in *Drosophila* and in *Mili* and *Miwi2* mutants in mouse. In *Drosophila*, the *Piwi* defect seems to be caused by a lack of the Piwi protein in somatic follicular cells that in turn causes depletion of germ-line stem cells (Cox et al. 2000). The *flamenco* mutation, which activates transposons in follicular cells, has a phenotype very similar to that of *Piwi*, indicating that *Piwi*-induced stem cell phenotypes can be explained by transposon misregulation. The activation of TEs in follicular cells might perturb their function, preventing them from providing the niche necessary for maintaining germ-line stem cells.

In mouse, *Mili* and *Miwi2* are expressed exclusively in germ cells, and the defects caused by these mutations seem to be cell autonomous (Kuramochi-Miyagawa et al. 2004; Carmell et al. 2007). Activation of transposons in stem cells themselves might be inconsistent with the long-term maintenance of a multipotent state. Although piRNA profiles in pure stem cell populations have not yet been characterized, it is plausible that stem-cell-maintenance defects are caused by transposon activation either in the stem cell itself or its niche cells, rather than by another regulatory defect. Transposon activation can affect cellular physiology in many different ways. For example, transposition can generate dsDNA breaks that are sensed by DNA-repair machinery as described above. Alternatively, abundant TE transcripts might impact the expression of normal cellular mRNAs. Finally, as described below, activation of transposons in mouse correlates with demethylation of their genomic sequences. Therefore, it is plausible that failures in genome-wide methylation of TEs perturbs the normal chromatin landscape of stem cell.

THE PIWI/piRNA PATHWAY AS AN EPIGENETIC SENSOR AND MEMORY IN GERM CELLS

As compared to miRNAs and siRNAs, the Piwi/piRNA pathway has several features that make it suitable as a mediator of epigenetic memory in germ cells. First, the pathway is not linear but instead includes a self-perpetuating loop called the ping-pong cycle (Aravin et al. 2007a; Brennecke et al. 2007). In the ping-pong cycle, primary piRNAs, generated by an unknown mechanism, can be amplified if complementary transcripts are available. Amplification initiates when the endonucleolytic activity of Piwi proteins is used to cleave complementary tran-

scripts. This induces the formation of a new, secondary piRNA with its 5′ end precisely at the cleavage site. The secondary piRNA can in turn regenerate the initial piRNA by cleavage of its complementary target. In *Drosophila*, the majority of primary piRNAs are derived from piRNA clusters and are antisense to transposon mRNAs (Brennecke et al. 2007). Therefore, on recognition of a transposon, they cleave its transcript and generate a sense secondary piRNA. Although the original silencing program is hardwired within the sequence of the piRNA clusters, amplification of each individual piRNA sequence depend on the existence of complementary transcripts, that is, on transcription of particular TEs. Thus, the system is able to sense expression of specific types of TEs and amplify piRNAs able to target those elements that are active within a given animal. In mouse, transcripts of TEs themselves are recognized as a source for primary processing, and therefore, primary piRNAs are sense and secondary are antisense, although the overall cycle operates similarly (Aravin et al. 2008).

Cycles that include production of secondary small RNAs are not restricted to the piRNA pathway (Baulcombe 2006). Indeed, situations in which production of secondary siRNAs depends on primary siRNAs or miRNAs have been described in fission yeast (Sugiyama et al. 2005), *C. elegans* (Pak and Fire 2007; Sijen et al. 2007), and plants (Allen et al. 2005; Axtell et al. 2006; Daxinger et al. 2009). Interestingly, in these organisms, piRNAs are either absent (yeast and plants) or produced exclusively by a primary processing mechanism without an amplification cycle (*C. elegans*). In all of these organisms production of secondary siRNAs depends on the activity of an RNA-dependent RNA polymerase (RdRP).

In plants, cleavage caused by primary siRNA or miRNA generates aberrant RNAs that are recognized as RdRP substrates (Allen et al. 2005; Axtell et al. 2006). In *C. elegans*, the primary siRNA somehow induces the RDRP to synthesize secondary siRNAs as direct transcription products (Pak and Fire 2007; Sijen et al. 2007). Secondary siRNAs can also recognize original transcripts, at least in some cases, closing the loop that amplifies the pool of small RNAs. In RdRP-dependent siRNA amplification mechanisms, secondary siRNAs spread from the site of primary siRNA, either in the 5′ direction (*C. elegans*) or both the 3′ and 5′ directions. Currently, there is no evidence of spreading during ping-pong amplification of piRNAs.

Amplification of piRNAs in the ping-pong cycle allows fine-tuning of piRNA populations to repetitive elements expressed in the cell and perpetuation of piRNA pools over cellular and even organism (in *Drosophila*) generations. Perpetuation of piRNA pools establishes a new system of epigenetic memory, completely independent of DNA/chromatin modifications (Brennecke et al. 2008). Furthermore, at least in mouse, there is clear evidence that piRNAs are linked to more classical epigenetic pathways that store information in the form of DNA methylation patterns (Aravin et al. 2007b, 2008; Carmell et al. 2007; Kuramochi-Miyagawa et al. 2008).

In mouse, it is well established that transposons are repressed transcriptionally, in a manner that depends on DNA methylation. Derepression of TEs in *Mili*- and *Miwi*2-deficient animals correlates with a failure of de novo methylation of these elements in the genome (Aravin et al. 2007b, 2008; Carmell et al. 2007; Kuramochi-Miyagawa et al. 2008). Several lines of evidence support a direct, functional link between the piRNA pathway and DNA methylation. The ping-pong cycle is most active during prenatal development, when patterns of DNA methylation on TEs are established (Aravin et al. 2008). At that time, two Piwi proteins are involved in the cycle: MILI and MIWI2. Expression of *Miwi2* precisely corresponds to the window of de novo DNA methylation in male germ cells, and this protein is present in the nucleus primed with small RNAs corresponding to mobile elements. Therefore, antisense piRNAs that are abundant in MIWI2 complexes might serve as sequence-specific guides that find and mark genomic sequences of transposons for DNA methylation. De novo DNA methyltransferases as well as *Miwi2* are not expressed after birth and the methylation pattern of TE sequences established in embryogenesis are maintained in germ-line stem cells. Similar patterns persist after fertilization and after the remodeling of the epigenome that occurs during early development. Because TEs constitute approximately 40% of genome in mouse, changes in their methylation patterns might have a substantial impact on the entire chromatin landscape of the cell, particularly if many components of heterochromatin normally exist in a tightly controlled balance with the sites that they regulate. Thus, although it is clear that loss of the piRNA pathway can impact DNA methylation states of transposons, this might also have secondary effects on broader gene expression patterns.

CONCLUSIONS

Small RNA pathways appear to be of particular importance in pluripotent and multipotent cells types. This relies on both inclusion and exclusion. Specific classes of miRNAs are often present in stem cell populations, and these may have critical roles in maintaining "stemness." Indeed, reprogramming experiments use lin-28, a regulator of miRNAs biogenesis, to aid in the conversion of fate-restricted to pluripotent cells (Yu et al. 2007; Darr and Benvenisty 2008). In this case, lin-28 is known to repress maturation of the let-7 family, a group of miRNAs that show widespread expression in differentiated cells (Heo et al. 2008; Newman et al. 2008; Viswanathan et al. 2008). Germ cells have greatly elaborated small RNA pathways, particularly in mammals. Germ cells are the only known cellular context in which all three extant RNAi-related pathways operate, the siRNA, miRNA, and piRNA pathways. Evidence from several animal models suggests that all three pathways are essential for germ cell integrity, with small RNAs serving to both control gene expression patterns and guard the integrity of the germ cell genome. Oocytes particularly are loaded with a variety of small RNAs during their maturation (Watanabe et al. 2006, 2008; Tam et al. 2008). At least in *Drosophila*, these maternally inherited species can have profound impacts on progeny, impacts that are also transmitted

through subsequent generations via the maternal lineage (Brennecke et al. 2008). Thus, small RNAs may not only be critical for germ cell maintenance but may form a mechanism for epigenetic inheritance.

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