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Small RNA in the nucleus: the RNA-chromatin ping-pong

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Abstract

Eukaryotes use several classes of small RNA molecules to guide diverse protein machineries to target messenger RNA. The role of small RNA in post-transcriptional regulation of mRNA stability and translation is now well established. Small RNAs can also guide sequence-specific modification of chromatin structure and thus contribute to establishment and maintenance of distinct chromatin domains. In this review we summarize the model for the inter-dependent interaction between small RNA and chromatin that has emerged from studies on fission yeast and plants. We focus on recent results that link a distinct class of small RNAs, the piRNAs, to chromatin regulation in animals.

Introduction

Eukaryotic cells have distinct chromatin domains that play crucial roles in the regulation of gene expression as well as maintenance of chromosome architecture in interphase and throughout cell division. Early studies suggested that chromatin was separated into densely packed repressive heterochromatin and less condensed active euchromatin; however, recent genome-wide profiling of chromatin states have revealed greater complexity of chromatin domains [1]. How different domains are established and maintained at specific genomic loci is largely unresolved. Binding of sequence-specific transcription factors and other DNA-binding proteins can lead to local recruitment of chromatin-modifying enzymes and structural chromatin proteins that establish distinct chromatin domains [2–6]. It is likely that other factors are also involved, especially as specific chromatin domains are established over extended genomic regions.

Like sequence-specific DNA-binding proteins, RNA molecules can guide chromatin factors to specific sequences. Indeed, the complementary interaction between guide RNA and unwound DNA or nascent transcripts provides perfect sequence-specific recognition. Long non-coding RNAs (lncRNA) such as Xist and HOTAIR appear to be responsible for targeting chromatin factors to specific domains [7–10]. Recently, the occupancy of several lncRNAs on chromatin was defined in a genome-wide survey [11]. However, it is still not clear if lncRNAs are able to directly recognize chromatin target sites through interactions with DNA or with nascent RNA ranscripts. In contrast to lncRNA, small RNAs between 20

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and 30 nt in length are too short to provide a scaffold for binding of several proteins or confer enzymatic activity, however, they are sufficiently long for highly specific recognition of complementary nucleic acid sequences.

Initially small RNA pathways were discovered in the artificial phenomenon of RNA interference (RNAi), the sequence-specific destruction of mRNA guided by small interfering RNA (siRNA) processed from long double-stranded RNA precursors introduced into the cell [12,13]. Later endogenous microRNAs (miRNAs) were found that regulate stability and translation of their mRNA targets through the same RNAi pathway [14–17]. Though initial studies showed that both siRNA and miRNA are involved in post-transcriptional repression of complementary RNA targets, studies in yeast, ciliates and plants implicated certain small RNAs in guiding modification of chromatin structure in the nucleus [18–25]. Here we briefly describe findings from studies of these species and discuss recent works that show that small RNAs regulate chromatin structure in animals.

Regulation of chromatin structure by small RNA in fission yeast and plants

Genetic and biochemical studies reveled that at the core of diverse small RNA pathways is a member of the Argonaute protein family and an associated small RNA that together form the RNA-induced silencing complex (RISC). Argonaute proteins have an RNAse H-like domain that is capable of endonucleolytic cleavage of RNA targets upon their recognition by the small RNA. In addition to the direct destruction of mRNA by the nuclease activity of Argonaute protein itself, RISC can recruit other proteins to the target mRNA to provide additional functions. For example, association of metazoan Argonautes with GW182 leads to relocation of mRNA to special cytoplasmic compartments, P-bodies, while recruitment of histone methyltransferase Clr4 in yeast induces local chromatin modification (see below). Knock-out of Argonaute proteins leads to elimination of associated small RNAs and provides direct insight into the function of the pathway.

The fission yeast *Schizosaccharomyces pombe* has a single Argonaute protein, Ago1, which associates with siRNAs and plays a crucial role in the establishment of pericentromeric heterochromatin [23]. This process involves several steps that together form a loop where chromatin and small RNA mechanisms reinforce each other (Fig. 1A) [18,26]. Repetitive pericentromeric sequences are bi-directionally transcribed generating double-stranded RNA (dsRNA) that are processed by the endonuclease Dicer to generate siRNAs, which bind to Ago1 [23]. The Ago1 complex, called RITS (RNA-induced transcriptional silencing) in *S. pombe*, is targeted to the pericentromeric chromatin by complementarity of the associated siRNA to the nascent transcripts [27]. Next, RITS recruits the Clr4 histone methyltransferase leading to methylation of histone H3 lysine 9 and subsequent binding of the HP1 homolog Swi6, which results in heterochromatin formation [28]. In parallel, RITS attracts the RNA-dependent RNA polymerase Rdp1, which generates additional dsRNA from the locus [29]. In agreement with the role of small RNA in establishment and maintenance of special chromatin structures, knock-out of Ago1 and Dicer lead to a decrease of H3K9 methylation and Swi6 binding at pericentromeric repeats [23]. Further studies revealed that production of siRNA is decreased in Clr4 mutants [28], while Swi6 was shown to be required for localization of Rdp1 [26]. Such a feedback can be explained by the fact that RITS contains a Chp1 chromodomain protein that recognizes methylated H3K9 [27]: Cooperativity between siRNA- and H3K9me-dependent interactions provides tethering of RITS complex to local chromatin.

Like those from fission yeast, plant small RNAs complex with specific members of the Argonaute family and are involved in formation of repressive chromatin structure through the process of RNA-directed DNA methylation [20]. In the *Arabidopsis* genome, DNA

methylation is concentrated in centromeric and repetitive regions and the same regions produce siRNA [30]. Precursors of siRNAs are transcribed by the plant-specific RNA polymerase IV and, in a mechanism similar to that in yeast, are converted into siRNAs by the activity of an RNA-dependent RNA polymerase and Dicer [31,32]. After binding to Argonaute AGO4, siRNAs recognize nascent transcripts produced by RNA polymerase V and recruit a DNA methyltransferase leading to DNA methylation at target loci [33–35]. This mechanism implies that target loci must be transcribed by pol IV and V for effective siRNA-mediated targeting. Chromatin structure likely plays a key role in recruitment pol IV and V to these loci.

The studies in fission yeasts and plants illustrate the complexity of the link between small RNA and chromatin silencing. In both systems, small RNAs are processed from precursors that are transcribed from loci that are the targets of regulation. Furthermore siRNAs recognize nascent transcripts and not DNA sequence. Accordingly, target loci must be transcribed in order to maintain a “silenced” chromatin structure. This concept is different from earlier models of transcriptionally-inactive heterochromatin. In both yeast and plants, the small RNA and chromatin structure are linked by a feed-forward loop: Small RNAs are necessary for establishment of specific chromatin marks, while specific chromatin structure is necessary to generate small RNAs through chromatin-dependent recruitment of the RITS complex (in yeast) or RNA pol IV and V (in plants).

Small RNA and chromatin in animals

The link between small RNA and chromatin pathways is less certain in animals than it is in plants and yeast. Indeed, in animals, siRNA and miRNA pathways seem to function primarily in the cytoplasm in post-transcriptional degradation and translational repression of target mRNA. Nevertheless, mammalian Argonautes shuttle to the nucleus [36]. In addition, exogenous siRNAs induce sequence-specific chromatin changes in mammalian cells [37], although whether this pathway is endogenously utilized by the cell remains to be established.

Though it seems that siRNAs and miRNAs are not directly involved in regulating chromatin structure in animals, other classes of small RNA might be involved. Indeed, a specific set of ~22 nt RNAs in *C. elegans* known as 22G-RNA that are antisense to thousands of germline-expressed protein-coding genes bind to a germline specific Argonaute, Csr1, which plays a role in chromosome cohesion in meiosis and mitosis likely through its effect on histone methylation [38]. Animals also have another conserved class of small RNAs, piwi interacting RNAs (piRNAs), that are implicated in chromatin regulation. In contrast to siRNAs and miRNAs, piRNAs are generated by a Dicer-independent mechanism [39] and associate with members of the Piwi clade of Argonaute proteins, which are expressed exclusively in germline [40–42]. The conserved function of the piRNA pathway is the repression of repetitive genomic sequences, including transposons, resembling the function of siRNA pathways in yeasts and plants. However, in contrast to yeast siRNAs that act in *cis* i.e. induce modification of chromatin structure at the same loci they are transcribed from, piRNAs in animals can act in *trans* to target multiple copies of transposable elements located in different genomic positions. Recent studies suggest that generation of piRNA from genomic loci is regulated by chromatin structure, similar to regulation of siRNA biogenesis in yeast.

The role of chromatin in determining piRNA expression

piRNAs are processed by an unknown mechanism from long single-stranded precursors transcribed from extended genomic regions called piRNA clusters [40–42]. piRNA precursors and mature piRNA do not appear to share sequence or structural motifs, and an

unanswered question is what features discriminate these RNAs from the rest of the transcriptome and destine them for piRNA processing. It is possible that, as in yeasts and plants, a special chromatin structure of piRNA clusters recruits factors that are necessary for piRNA processing. Indeed, major piRNA clusters in *Drosophila* are localized at telomeres and pericentromeric regions on the borders between gene-rich euchromatic areas and centromeric heterochromatin [43].

Study of piRNA clusters in *Drosophila* showed that they indeed have heterochromatic structure based on HP1 binding and position-effect variegation of transgenes inserted into these regions [44]. Early during oogenesis, discrete foci are observed in germline nuclei that contain telomeric and centromeric regions, where all major piRNA clusters are located [45**]. These foci are enriched in repressive histone H3 lysine 9 and histone H4 lysine 20 tri-methylation marks.

The first indication that chromatin structure of piRNA clusters is indeed important for generation of piRNA was obtained by genetic dissection of the trans-silencing phenomenon in *Drosophila*. In trans-silencing, a transgene inserted in a telomeric piRNA cluster gives rise to piRNAs that can silence a homologous transgene inserted elsewhere in the genome [46–48]. Interestingly, piRNA-mediated trans-silencing is released by mutations in the piRNA pathway and by mutation in the gene encoding the heterochromatic protein HP1 that binds methylated H3K9 and is present at telomeres [47,49]. Recent studies showed that a specific chromatin structure is required for piRNA generation from other, non-telomeric piRNA clusters. Mutations in *rhino*, which encodes the germline-specific HP1 homolog, and *SetDB1*, the gene for the enzyme that methylates H3K9 to provide the target for HP1/Rhino binding, impairs production of piRNAs and leads to the collapse of the piRNA pathway [45**,50**]. A similar defect is observed in *Drosophila* with mutations in Cutoff protein. Cutoff has a similarity to the yeast transcription termination factor, its binding is enriched on piRNA cluster chromatin, and it interacts with Rhino [51**]. Interestingly, three proteins required for efficient piRNA generation, HP1, Rhino and SetDB1, are thought to be hallmarks of “repressive” chromatin (Fig. 1B).

Distinct localization of piRNA clusters at telomeres and pericentromeric regions in *Drosophila* suggest that these genomic regions have special properties necessary for piRNA generation. However, when piRNA clusters are moved to ectopic locations as a transgenes they retain their ability to generate piRNAs, demonstrating that genomic position *per se* is not crucial [48*] (Fig. 2). It remains to be tested whether transgenic piRNA clusters moved to ectopic loci maintain their heterochromatic structure at euchromatic positions.

Overall, studies in flies suggest that, as in yeast and plants, chromatin marks that are considered to be ‘repressive’ are necessary for active transcription of piRNA precursors from piRNA clusters (Fig. 1B). It remains to be elucidated whether other marks are involved in specifying regions that produce piRNAs: Although the effect of Rhino deficiency seems to be specific for piRNA clusters and not other heterochromatic sequences [50**], the trimethyl-H3K9 mark is not restricted to loci that generate piRNA. Furthermore, future studies will need to address both necessity and sufficiency of specific chromatin marks in marking loci for piRNA generation.

The role of piRNAs in regulating chromatin structure

Like Argonautes that bind other small RNAs, the Argonaute-like Piwi proteins have piRNA-guided endonuclease activity that can efficiently degrade targets such as mRNAs of transposable elements. However, post-transcriptional silencing guided by piRNA does not exclude a role in regulation of chromatin structure. Indeed both flies and mice have three Piwi proteins and in each species one protein localizes to the nucleus [52,53]. Genetic data

unequivocally implicate piRNAs in establishing *de novo* DNA methylation patterns in the mouse male germline [52,54,55].

Mammalian germ cells erase and reestablish DNA methylation patterns during embryogenesis after segregation from somatic cells. The failure to methylate sequences of retrotransposons scattered throughout the genome leads to their activation and subsequent meiotic failure and sterility [56]. The defects observed in *Dnmt3L* knock-out animals that are deficient in *de novo* DNA methylation is surprisingly similar to those observed in animals deficient in two Piwi proteins, MILI and MIWI2. Furthermore, methylation patterns were not reestablished on retrotransposon sequences in mice lacking Mili or Miwi2 [55]. Both proteins are loaded with piRNAs that target transposon sequences and MIWI2 is exclusively expressed and localizes to the nucleus in the short developmental window when *de novo* methylation occurs in male germ cells [52] (Fig. 3A). MILI itself localizes to the cytoplasm, and its expression is necessary for MIWI2 nuclear localization. Male germ cells also establish new methylation patterns at imprinted loci. The piRNA pathway is involved in methylation of at least one imprinted locus, *Rasgrf1* [57*]. This process depends on a solo-LTR located in the *Rasgrf1* locus and might be a consequence of piRNA-mediated retrotransposon methylation.

It seems that piRNA provide a sequence-specific guide for methylation of genomic sequences of active transposons through recognition of nascent transcripts. Biochemically this process remains unexplored due to restriction of *de novo* DNA methylation to a small number of germ cells in a narrow developmental window during embryogenesis. It is possible that DNA methylation is not the primary effect induced by the Piwi-piRNA complexes and that piRNAs first guide changes in histone marks, which are recognized by the DNA methylation machinery [58] (Fig. 3B).

In contrast to mammals and plants, in *Drosophila* DNA methylation does not play a major role in regulation of chromatin structure and gene expression. Nevertheless, the presence of Piwi in the nucleus suggests its involvement in regulating chromatin structure. Cell culture experiments show that deletion of Piwi's nuclear localization signal leads to failure of transposon silencing even though Piwi proteins are loaded with piRNAs in the cytoplasm [59].

The specific role of Piwi in the nucleus remains controversial. Early studies demonstrated effects of Piwi and other piRNA pathway genes on chromatin-mediated gene silencing and genome-wide distribution of several histone marks [60–62]. However, the significance of these observations remains to be clarified as effects are observed in somatic cells where the piRNA pathway is not active and in heterozygous animals that do not show defects in transposon silencing in the germline. Piwi directly interacts with HP1 [63]; however, *piwi* knockdown does not affect HP1 association with piRNA clusters [44]. Other studies suggested that at least some transposable elements are transcriptionally repressed by the piRNA pathway. Particularly, transcription of several retrotransposons, including the telomere-specific element HeT-A, is significantly increased in piRNA pathway mutants as seen by nuclear run-on assays in *Drosophila* ovaries [64*]. Interestingly, telomeric retrotransposons also show nuclear accumulation at sites of transcription in piRNA pathway mutants [65,66]. Additionally, slight increases in active marks (dimethylation of H3K79 and H3K4) and decreases of repressive marks (di/trimethylation of H3K9) are observed for several transposons in piRNA pathway mutants in which the majority of piRNAs are eliminated [64*,67]. Additional studies are clearly needed to investigate the role of the piRNA pathway in regulation of chromatin structure.

Conclusions

Small RNA pathways provide an ingenious modular mechanism that allows separation of target recognition and effector functions into two different components of one ribonucleoprotein complex. Not only do small RNAs provide highly specific recognition of complementary targets, the pathway also provides the flexibility to produce new small RNAs to recognize additional targets. The Argonaute protein components also provide a diversity of functions through interactions with additional proteins. It is not surprising that small RNA pathways are widespread in eukaryotes and regulate many aspects of gene expression. It is estimated that mammalian miRNAs post-transcriptionally regulate the expression of more than 30% of human genes [68]. Though no direct binding of small RNA to DNA has yet been shown, the role of small RNA pathways is not restricted to post-transcriptional regulation, as binding to nascent transcripts can effectively mediate sequence-specific recruitment of small RNA-Argonaute complexes to chromatin.

Single celled eukaryotes such as fission yeasts and ciliates as well as plants successfully employ small RNA pathways to maintain specific chromatin domains. The common theme that has appeared from studies in these organisms is that there is a tight link between small RNA and chromatin-modification pathways in a self-reinforced loop. The paradox of this mechanism is that transcription of target loci is necessary in order to maintain both small RNA production and chromatin marks that were believed to be ‘repressive’.

Although direct activity of miRNA and siRNA in animals seems to be restricted to post-transcriptional regulation of protein-coding genes, another class of small RNAs, piRNAs are necessary for silencing of repetitive genomic elements in germ cells – likely through both transcriptional and post-transcriptional mechanisms. Recent studies in *Drosophila* demonstrate that, as in yeast and plants, ‘repressive’ chromatin marks on piRNA-producing genomic loci are required for their generation indicating a two-way communication between the piRNA pathway and chromatin.

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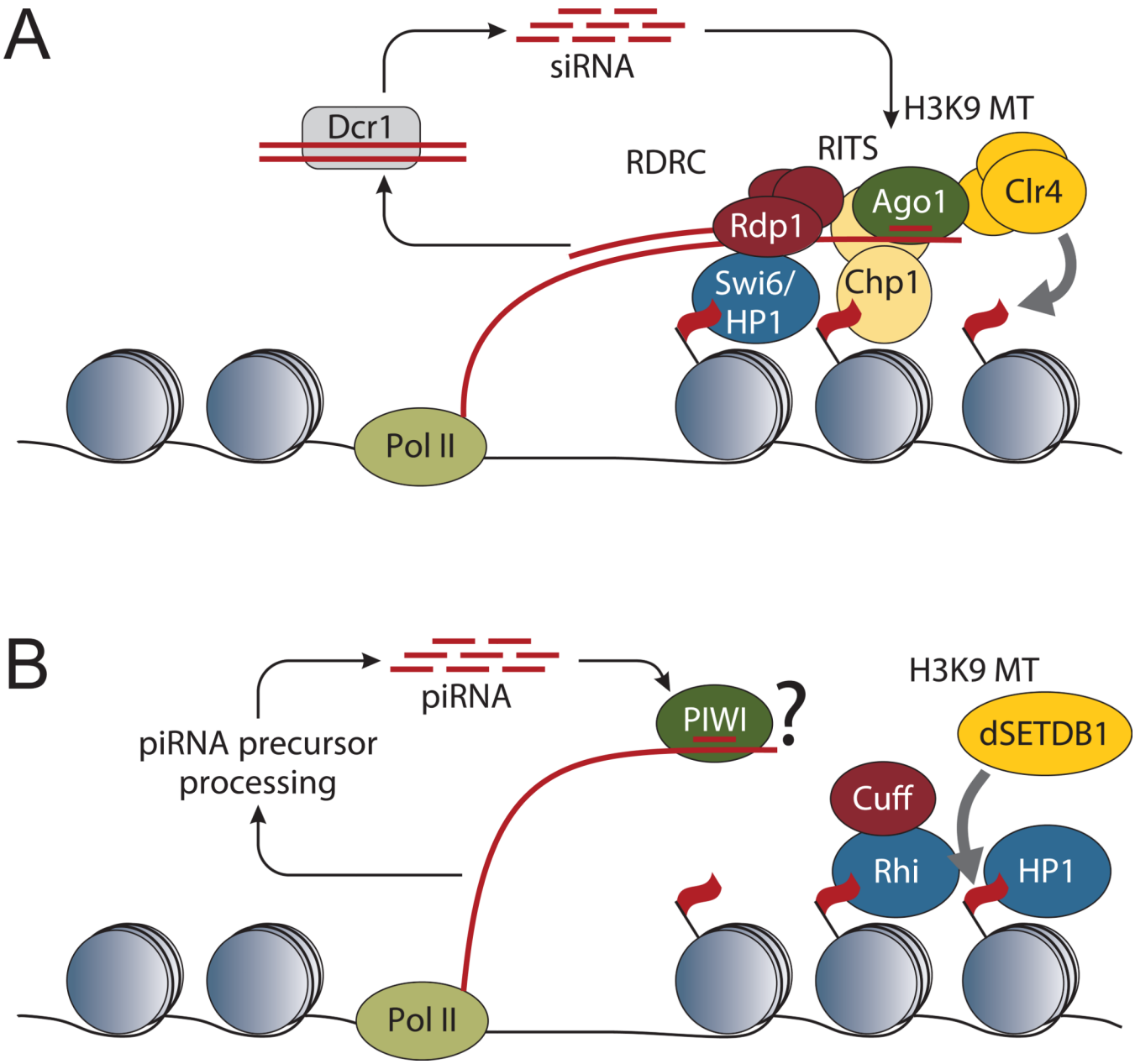

Figure 1. The link between small RNA production and chromatin in fission yeast and *Drosophila*

(A) In yeast, repetitive pericentromeric sequences give rise to dsRNAs that are processed by the endonuclease Dicer to generate siRNAs, which bind to Ago1 in the RITS complex. RITS can be recruited to chromatin through two, probably cooperative, mechanisms: First the siRNA provides the specificity for the RITS interaction with nascent transcripts. Second, RITS contains Chp1 that binds methylated H3K9 (red flag). RITS attracts the RNA-dependent RNA polymerase Rdp1, which generates additional dsRNA, and histone methyltransferase Clr4 that methylates histone H3 at lysine 9 providing a binding site for the HP1 homolog Swi6. Note that RITS induces methylation of H3K9 and the same mark enhances its binding to chromatin.

(B) In *Drosophila*, piRNAs are processed from long single-stranded precursors transcribed from piRNA clusters. Four proteins present on chromatin at piRNA clusters are necessary

for effective piRNA generation: the histone methyltransferase dSetDB1 that methylates histone H3 at lysine 9 (red flag), HP1 and its homolog Rhino that bind methylated H3K9, and Cuff that interacts with Rhino. In germ cells, mature piRNAs are loaded into three Piwi proteins; two Piwi proteins localized exclusively in the cytoplasm and a third, Piwi itself, that shuttles to the nucleus and has a potential to recognize nascent transcripts through a piRNA-mediated interaction (question mark).

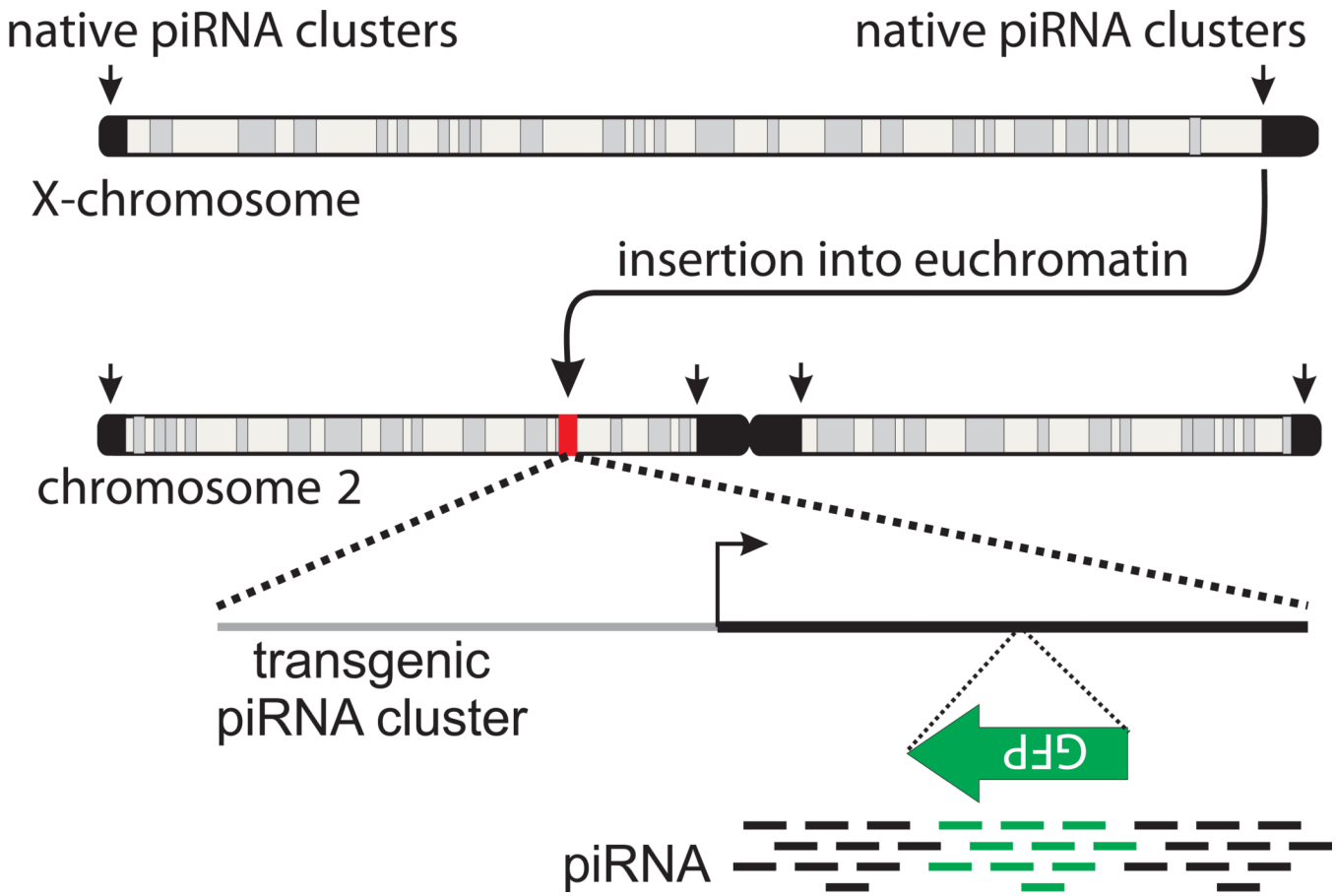

Figure 2. The genomic position of piRNA clusters in *Drosophila*

The major piRNA clusters in *Drosophila* are localized at telomeres and pericentromeric regions on the border between gene-rich euchromatic areas (grey) and centromeric heterochromatin (black). Clusters can be inserted as BAC transgenes into heterologous euchromatic positions. The activity of transgenic cluster is demonstrated by generation of piRNA from an artificial sequence (GFP) inserted in the transgene.

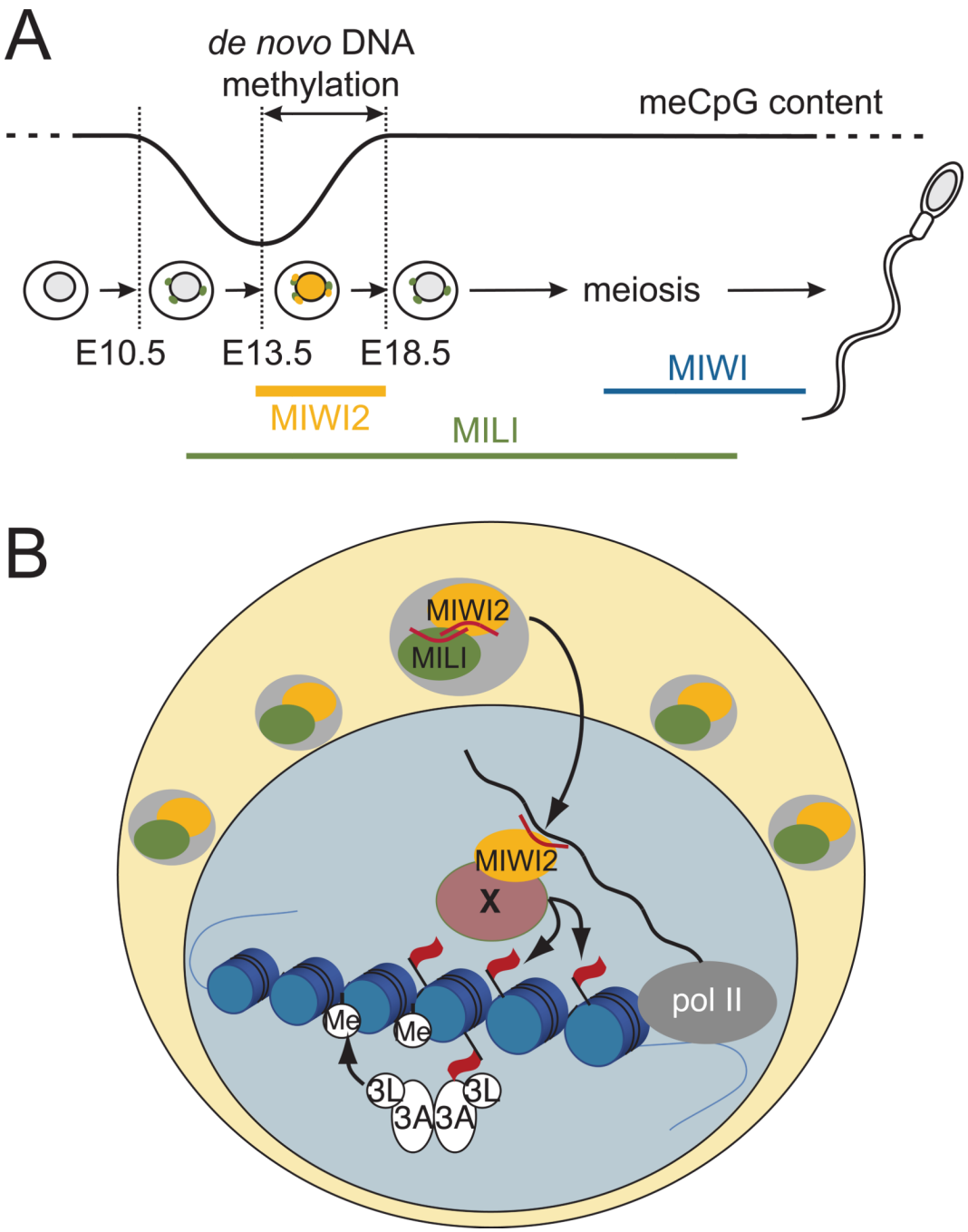

Figure 3. The role of piRNAs in *de novo* DNA methylation in mammalian germ cells

(A) In mouse, the germ cells are segregated from somatic cells at day six of embryonic development (E6.5) followed by erasure of old methylation patterns between E9.5 and E12.5 and establishment of new CpG methylation between E13.5 and E18.5. Of the three murine Piwi proteins, MILI, MIWI and MIWI2, only MIWI2 is localized in the nucleus and expressed during the time window of *de novo* methylation.

(B) The putative mechanism of piRNA-guided DNA methylation. Two Piwi proteins, MILI and MIWI2, localize to cytoplasmic granules and participate in the ping-pong mechanism that amplifies piRNAs that target active transposable elements. The loading of MIWI2 with piRNA is dependent on MILI function. The MIWI2-piRNA complex translocates to the

nucleus and binds to nascent transposable element transcripts followed by recruitment of unknown chromatin modifiers (X). Subsequent changes in histone marks (loss of H3K4 methylation and gain of H3K9 methylation, red flags) induce the activity of the Dnmt3A–Dnmt3L tetrameric methylase complex to establish CpG methylation patterns.