NON-CODING RNA

PIWI-interacting RNAs: from generation to transgenerational epigenetics

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Abstract | Small-RNA-guided gene regulation is a recurring theme in biology. Animal germ cells are characterized by an intriguing small-RNA-mediated gene-silencing mechanism known as the PIWI pathway. For a long time, both the biogenesis of PIWI-interacting RNAs (piRNAs) as well as their mode of gene silencing has remained elusive. A recent body of work is shedding more light on both aspects and implicates PIWI in the establishment of transgenerational epigenetic states. In fact, the epigenetic states imposed by PIWI on targets may actually drive piRNA production itself. These findings start to couple small RNA biogenesis with small-RNA-mediated epigenetics.

PIWI-interacting RNAs (piRNAs) are small, 21–30 nt single-stranded RNAs that associate with PIWI proteins in various organisms. Typically, multiple PIWI paralogues are encoded in a given organism, and the family is named after the initially identified member, Piwi, in *Drosophila melanogaster*[1,2]. PIWI proteins are a clade within the larger family of Argonaute proteins that is mostly specifically expressed in the germ line[3–5]. In general, Argonaute proteins use their bound small RNA molecules to identify relevant RNA targets through base-pairing interactions. The precise function of an Argonaute at a specified target can be quite diverse, but usually the end result is silencing of the target. The silencing by Argonaute proteins can proceed through target RNA degradation (using the RNase H-like domain of Argonaute proteins[4–9]), the inhibition of translation or the recruitment of chromatin-modifying activities[10–14]. Generally, to exert a function on targeted RNAs, Argonaute proteins recruit additional cofactors[3].

In most animal species studied, PIWI proteins have been shown to repress 'non-self' sequences, such as transposable elements[15]. These elements have the ability to move and/or to multiply themselves to new positions in the genome, thereby posing a threat to the genomic stability of an organism[16]. However, non-transposon-related PIWI targets and piRNAs derived from regular mRNAs have been described as well, including early developmental genes and genes involved in the establishment of memory in neurons[17–21]. These aspects will not be discussed here further. We focus on recent developments on the molecular mechanisms of the PIWI pathway, including piRNA biogenesis and target silencing.

The biogenesis of piRNAs is quite different from that of other small-RNA pathways, such as the microRNA (miRNA) pathway. In the miRNA pathway, the endonuclease Dicer, which cleaves double-stranded RNA, is essential, whereas for piRNAs, Dicer is dispensable[22,23]. Consistently, instead of double-stranded RNA molecules, single-stranded RNA molecules are used as precursors for piRNAs[24,25]. These transcripts are derived from specific loci named piRNA loci or piRNA clusters. The transcription factors involved are currently largely unknown. The piRNA precursors are then believed to be processed into mature piRNAs in the context of an electron-dense cytoplasmic material — known as nuage or inter-mitochondrial cement[26,27] — that can be found at the nuclear pores of germ cell nuclei. piRNA biogenesis occurs through both primary and secondary pathways[3,12,15], which are distinguished by the mechanism by which the 5′ ends of piRNAs are generated. The mechanism of piRNA precursor cleavage in the primary pathway has long been — and to some extent still is (see the discussion below) — an open question, whereas in the secondary pathway, it is mediated by the PIWI proteins themselves[24,28,29]. Functionally, primary biogenesis is thought to initiate PIWI pathways, whereas secondary biogenesis (sometimes also referred to as ping-pong amplification) is thought to ensure that the total pool of piRNAs relates well to the expression of the actual targets of primary piRNAs. This idea has

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been best documented in *D. melanogaster*, in which mutation of a gene encoding a Piwi protein that acts only in the secondary pathway leads to a collapse of the whole piRNA pool[30], and maternally inherited piRNAs have been shown to set-up zygotic piRNA populations[31,32]. For further reading on the background of these processes, see REFS 3,15,33,34.

At the level of target silencing by PIWI proteins, many mysteries remain. Some PIWI proteins act in the cytoplasm and may trigger degradation of mRNAs[22,24,29,30,35]. However, some PIWI proteins also translocate to the nucleus on piRNA loading, suggesting they may have a role in silencing their targets at the transcriptional level. Such phenomena are well described at the molecular level in non-piRNA pathways in, for example, fission yeast and plants[18,36–38]. However, until recently, it was unclear to what extent these molecular details were relevant for nuclear PIWI activity.

Recent publications have shed considerable light on the mechanisms of piRNA biogenesis and PIWI target silencing. In this Review, we focus on these recent advances and follow piRNA biogenesis from the transcription of precursor piRNA transcripts through to their nuclear export and processing into mature piRNAs and finally back into the nucleus, where PIWI proteins exert their functions relating to transcriptional gene silencing. We will see that piRNA-mediated silencing at genomic loci can remain stable through organismal generations and thus might represent a mechanism of transgenerational epigenetic inheritance. Furthermore, the resulting chromatin at silenced PIWI targets seems closely related to the chromatin found at piRNA-encoding loci, raising the intriguing possibility that the silencing activities of PIWI proteins may act both to silence targets as well as to specify loci for precursor piRNA transcription.

piRNA biogenesis

Transcription and transport of piRNA precursor RNAs. An important question is how are precursor piRNA transcripts selected to enter the piRNA-processing pathways? One scenario could be that transcription of precursor piRNAs has special features that lead to the specific uptake of these transcripts into piRNA biogenesis mechanisms. This scenario might operate in *Caenorhabditis elegans*. In this animal, most piRNA species (known as 21U RNAs)[39,40] are individually encoded by separate genes[41], and each is characterized by a conserved motif that is found ~40 bp upstream of the piRNA-encoding sequence[42] (FIG. 1a). The motif is an octamer sequence that is recognized by Forkhead family (FKH) transcription factors, which are required for the expression of 21U RNAs[43]. Transcription of these precursors starts two bases upstream of the mature piRNA 5′ end. The transcripts are then 5′ capped[43] and are mostly 26 nt in length[44]. Additionally, so-called 'type 2' 21U RNAs have been described in *C. elegans*; these 21U RNA species relate to the 5′ ends of full-length mRNAs[44], suggesting that 21U RNA biogenesis may also involve the generation of short 5′ transcripts from full-length genes. The molecular details behind the generation of both types of 21U precursors are currently unclear, but possibly they relate to mechanisms involved in premature transcriptional termination near the 5′ ends of genes[45].

However, the generation of single piRNAs from precursor transcripts seems not to operate in many other species. In most studied organisms, piRNAs are derived from precursor transcripts that are a few kilobases to more than 100 kb in length and are processed into many different piRNAs[22,24,46–49]. The promoters and transcription factors involved are starting to be identified: for example, in *D. melanogaster*, promoter mutations have been retrieved in a piRNA cluster that drives very long single-stranded transcripts[24], and in mice the transcription factor MYB-related protein A (A-MYB) has been shown to drive transcription of specific piRNA clusters[25]. How are such piRNA precursor transcripts distinguished from other long transcripts and sent into piRNA biogenesis routes? The answer to this question is currently not known, but one hypothesis is that a particular chromatin feature of piRNA-producing loci may have a role. This thought is largely based on the finding that in *D. melanogaster*, a particularly fast evolving homologue of heterochromatin protein 1 (HP1) called Rhino binds to many piRNA clusters and is required for their piRNA production[50]. But how can such a heterochromatin-related protein be required for the production of any RNA species, as it seems to contradict the generally accepted role for HP1 proteins in transcriptional silencing[51]? The answer may come from a recent study showing that HP1-bound loci in fission yeast can still be transcribed[52]. In that system, HP1 not only functions by binding histone H3 trimethylated on lysine 9 (H3K9me3) but also acts as an RNA-binding protein that accepts transcripts from its bound loci and transports these to sites at which they will be degraded. Thus, HP1 shuttles between chromatin-bound and chromatin-free states. Such a shuttling role for Rhino or other functionally related proteins could play a part in piRNA pathways: instead of targeting transcripts for degradation, Rhino might escort precursor piRNA transcripts to cellular sites of piRNA processing (FIG. 1b).

In fact, Rhino has been shown to colocalize with the DEAD box protein UAP56 in nuclear speckles bound to piRNA precursors, and it has been proposed that both proteins have important roles in specifying piRNA precursors and sending them to the right subcellular compartments for piRNA processing[23,24,35,46,48,49,53]. This processing is believed to occur in specialized nuage domains that flank nuclear pores on the cytoplasmic side. Indeed, UAP56–Rhino foci on the nuclear side of nuclear pores are often flanked on the cytoplasmic side by the nuage marker Vasa, which is another DEAD box protein[26,53]. UAP56 is proposed to act on the nuclear side to hold on to piRNA precursors until it is accepted by Vasa on the other side of the nuclear membrane (FIG. 1b). In support of this, mutations in *UAP56* that are presumably defective in RNA binding disrupt its colocalization with Rhino, disturb subcellular Vasa localization and inhibit piRNA production.

21U RNAs
Short RNA molecules found in *Caenorhabditis elegans* that are characterized by a length of mostly 21 nt and a uracil at their 5′ end. 21U RNAs are bound by the Piwi proteins PRG-1 and PRG-2.

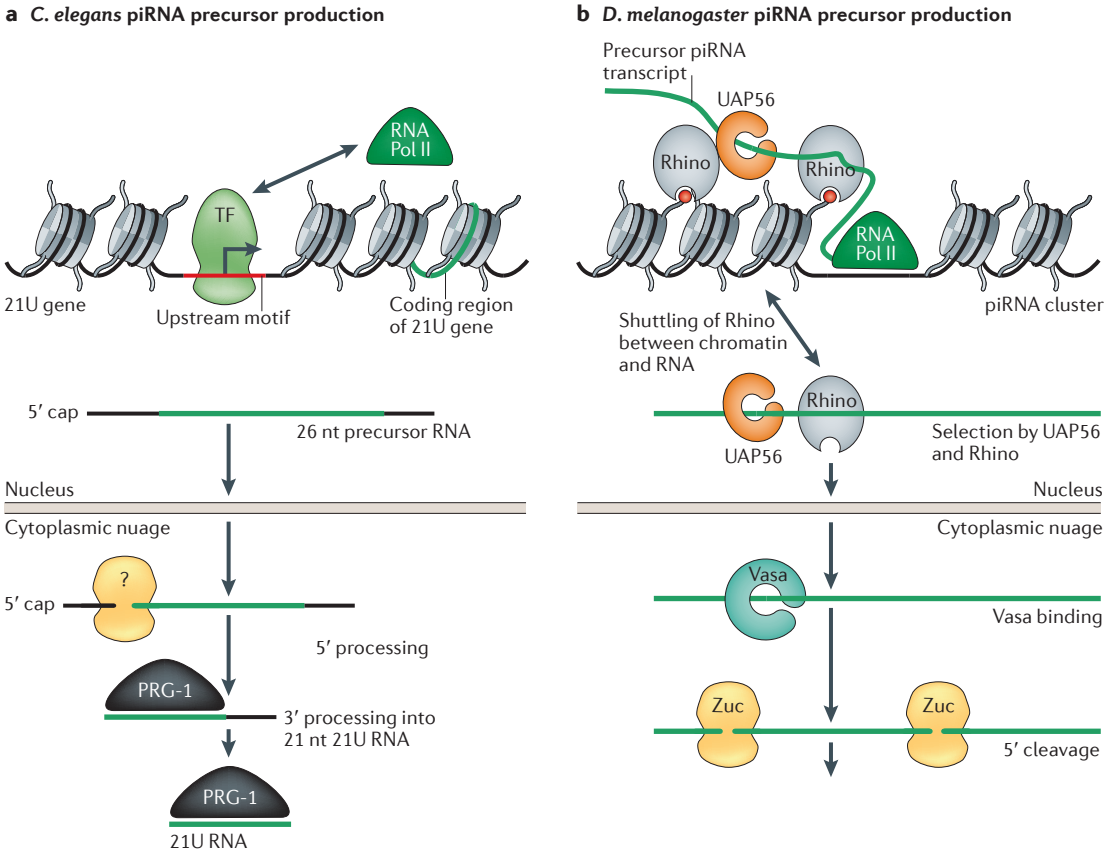

Figure 1 | **Transcription and selection of piRNA precursors.** **a** | In *Caenorhabditis elegans*, PIWI-interacting RNAs (piRNAs) are individually encoded by small transcription units characterized by consensus motifs (red DNA region) upstream of the piRNA-encoding DNA[42] (green DNA region). Forkhead transcription factors (TFs) have been shown to transcribe these loci[43], and the primary transcripts from these loci are mostly 26 nt in length[44]. How these small transcripts are terminated and how they are specified for 21U RNA production is unclear. Nevertheless, because the 26 nt precursors are two bases longer at their 5′ end than are the mature 21U RNA, we anticipate an endonucleolytic event that removes these two bases. **b** | In *Drosophila melanogaster*, loci that give rise to piRNAs may have a particular chromatin structure. In particular, a heterochromatin protein 1 (HP1)-like protein named Rhino has been identified to bind to many (but not all) piRNA-producing loci[50]. Rhino further interacts with the RNA helicase UAP56 (REF. 53) and with piRNA precursor transcripts. These transcripts then presumably channel through the nuclear pores and are captured on the cytoplasmic side by the nuage component Vasa, which is another RNA helicase. Transcripts coming from loci that lack Rhino-like proteins may not be able to follow the same route and hence will not be processed into piRNAs.

Although it is tempting to speculate that the previously described processes ensure that only piRNA precursors, rather than normal mRNAs, are delivered to sites of piRNA processing, clearly many predictions coming from this model still have to be experimentally tested. For example, why do some genic mRNAs give rise to piRNAs[19]? Would the tethering of Rhino or UAP56 to 'normal' transcripts turn them into piRNA precursors? And can Rhino homologues be identified in other species? Whatever the precise roles of Rhino and UAP56 are, another unresolved issue is how Rhino initially finds its target piRNA-encoding loci in the genome in order to serve as a molecular tag for these loci. Although this is currently unknown, there may be an intriguing link to the gene-silencing activities of the PIWI pathway itself, as will be discussed later.

5′ end formation. The next step in piRNA biogenesis is 5′ end specification. How does this work? In the secondary piRNA pathway, the 5′ end of piRNAs is generated by PIWI-induced cleavage as a part of the ping-pong mechanism (FIG. 2). However, in the primary pathway, this activity has long been disputed. A strong candidate to execute this step is a protein named Zucchini (Zuc) because in *D. melanogaster*, Zuc mutants display primary biogenesis defects[54], and on the basis of primary sequence, Zuc is a protein with potential nuclease activity. One of the reasons why the role of Zuc has been difficult to pin down is because besides its predicted nuclease domain, its primary amino acid sequence shows features that predict phospholipase activity.

One proposed function of Zuc in germ cells is to generate phosphatidic acid on the mitochondrial surface,

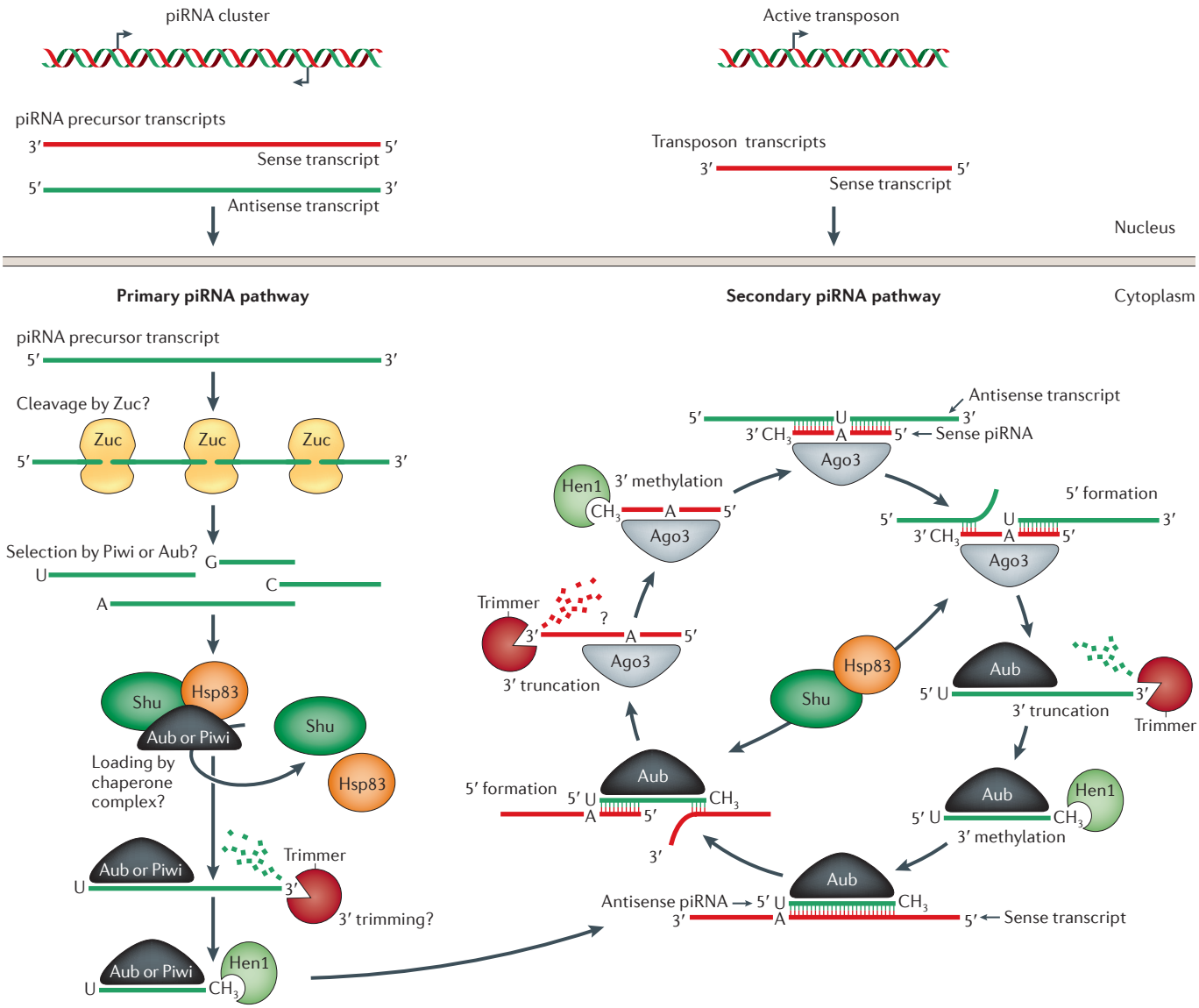

Figure 2 | **Primary and secondary piRNA biogenesis.** Here we depict the general features of both primary and secondary Piwi-interacting RNA (piRNA) biogenesis, using the Piwi pathways of *Drosophila melanogaster* as an example. In the primary pathway, the precursors are cleaved by an endonuclease, most likely Zucchini (Zuc)[59–61]. Next, cleaved fragments are incorporated into Piwi or Aubergine (Aub) with the help of Shutdown (Shu) and Heat shock protein 83 (Hsp83, which is the fly homologue of HSP90)[69]. Fragments with a 5′U may be heavily selected for at this step. After loading into the Piwi protein, the unknown Trimmer enzyme trims the 3′ end[63] to fit the Piwi protein, after which Hen1 (REFS 74,75) methylates the mature 3′ end. This completes primary biogenesis. Aub, but not Piwi, can then enter the secondary pathway. Aub can recognize a cognate transcript and cleave it. The 3′ cleavage fragment of the targeted RNA can then be taken up by another Piwi protein named Argonaute 3 (Ago3), again with the help of Shu[69]. In addition, on the basis of work in mice[72], Shu may assist in removing 5′ cleavage fragments following Aub cleavage. Further downstream, steps are probably identical to primary biogenesis. In turn, Ago3 may assist in loading more Aub protein with secondary piRNAs. Very similar processes take place during piRNA biogenesis in other species, such as mice and fish, although many details appear to be quite flexible. For example, in mice, primary piRNAs have sense polarity.

which would induce the recruitment or activation of Piwi-related nuage components. Consistent with this, Zuc is present on the membranes of mitochondria, and in mice that are deficient for ZUC (which is also known as PLD6 in mice), mitochondrial defects are observed[24,28,55–57]. Furthermore, studies based on *in vivo* relocation experiments using sensors that detect phosphatidic acid suggest that mouse ZUC is indeed a phospholipase[3,15,33,34,55,56], and one study showed such activity *in vitro*[58]. However, the crystal structures of mouse ZUC and *D. melanogaster* Zuc have now been determined, and their biochemical properties are more robustly characterized. These results clearly show that both proteins have the three-dimensional fold of

a nuclease, and *in vitro* they are capable of cleaving single-stranded DNA and RNA[59–61]. Although these data still leave space for additional roles of Zuc in the piRNA pathway, a role as an enzyme that cleaves single-stranded RNA seems evident. Whether this indeed is 5′ end generation remains to be definitively proven, but in the absence of stronger candidates, Zuc is the prime suspect to act as the 5′ nuclease during primary piRNA biogenesis.

However, in mice that are deficient for ZUC, low levels of processed piRNAs can still be detected[56]. How can this be reconciled with the idea that ZUC generates the 5′ ends, which is a seemingly essential step? The answer may simply be that ZUC merely boosts precursor cleavage without being absolutely essential for it. In the absence of ZUC, other nucleases may generate sufficient cleavages in piRNA precursors to generate the observed piRNAs. In this respect, it is noteworthy that *in vitro* this enzyme does not specifically seem to generate fragments with a 5′U base, which is the signature of primary piRNAs. Rather, this signature may be imposed later, through selective binding of PIWI proteins to piRNA intermediates that contain a 5′U (FIG. 2), just as Argonaute proteins in *Arabidopsis thaliana* have been shown to select small RNA species with a strong preference for specific 5′ bases[62]. We note that *C. elegans* does not appear to have a Zuc homologue, whereas 21U precursors are still in need of some 5′ end processing activity. This activity has so far remained totally unexplored. Finally, a potential piRNA biogenesis intermediate carrying a 5′ hydroxyl group has been described to accumulate in *D. melanogaster* mutants that are defective in piRNA loading. This species was also shown to carry a 2′–3′ cyclic phosphate[57]. How this species relates to the above and to the below is currently unclear.

PIWI loading. Following 5′ cleavage and preceding the generation of the mature 3′ end, it is thought that the piRNA intermediates are loaded into PIWI proteins (FIG. 2). This is based on *in vitro* piRNA loading data[63] as well as on the characteristics of piRNA 3′ ends (see below). Several studies in both animals as well as plants have indicated that the loading of RNA molecules into Argonaute proteins is facilitated through the action of chaperone proteins, most notably heat shock protein 90 (HSP90) homologues[64–68]. A study in *D. melanogaster* has now shown that a protein named Shutdown (Shu) — a chaperone of the immunophilin class that contains a tetratricopeptide repeat (TPR) and a prolyl-isomerase domain — is required to load piRNA intermediates into PIWI proteins as well[69]. In order to do so, Shu requires its TPR domain to mediate interaction with Hsp83 (the fly orthologue of HSP90), which was previously shown to affect piRNA biogenesis and transposon silencing[70]. In *D. melanogaster*, the primary piRNA biogenesis pathway can be cleanly separated from the secondary simply by analysing the germline follicle cells that use only the primary pathway, hence it could be demonstrated that Shu is essential for both piRNA biogenesis pathways[69]. This shows that following the distinct events during 5′ end generation, primary and secondary piRNA biogenesis pathways seem to merge into a common mechanism during loading of piRNA intermediates.

Shu is a conserved protein, and the mouse homologue FKBP6 has also been found to be associated with PIWI proteins[71,72]. Furthermore, FKBP6-deficient mice display similar fertility defects to those observed in mice that are deficient for the PIWI proteins MILI and MIWI2. However, on molecular dissection of defects in FKBP6-deficient mice, no defect in primary piRNA biogenesis was observed[72]. Rather, the authors of this work suggest that FKBP6 is required only for the biogenesis of MIWI2-bound piRNAs, which arise solely through secondary biogenesis. Still, like *D. melanogaster* Shu, mouse FKBP6 was found to interact with HSP90 via the TPR domain, suggesting that at least some of its roles in the PIWI pathway are conserved. On the basis of experiments with *Bombyx mori* ovary-derived cells in culture and pharmacological inhibition of Hsp90 with geldanamycin, it was suggested that the Hsp90 machinery is required to remove one of the cleavage fragments from Piwi–target RNA complexes following target cleavage[72].

Thus, seemingly, two different roles for FKBP6–HSP90 complexes in the PIWI pathway have been put forward. One is a general role during piRNA loading, and one entails a function specific to secondary biogenesis. How can these be reconciled? One explanation is that Shu and FKBP6 execute both functions but that the role of FKBP6 in secondary biogenesis can be seen only in specific circumstances. Such conditions could be that the role of FKBP6 during piRNA loading in mice may be redundant, whereas its role during secondary biogenesis is not. Likewise, pharmacological inhibition of HSP90 may not have a major impact on primary biogenesis if we assume that at the time of HSP90 inhibition, a sufficient pool of piRNA-loaded PIWI protein is present, such that only the role of FKBP6 during secondary biogenesis can be revealed. Finally, how exactly FKBP6 or Shu functions is not known. For example, mammalian MOV10L1 (known as Armi in *D. melanogaster*) is an RNA helicase that has also been implicated in piRNA loading, but it is still unclear how FKBP6 and HSP90 interact with this protein.

3′ end formation. The last step in piRNA biogenesis involves the formation of its 3′ end (FIG. 2). An important clue to the formation of the piRNA 3′ end came from analyses of piRNA length profiles. In most species, the length distribution of piRNAs is relatively broad, and there is greater variability at the 3′ end than at the 5′ end. So far, only *C. elegans* piRNAs deviate from this picture, as for unknown reasons 21U RNAs display a much more strict size distribution[42]. In all other species studied, the typical size profile of piRNA populations shows bell-shaped distributions with a maximum length that is characteristic of specific PIWI paralogues. For example, the three Piwi proteins in *D. melanogaster* — Piwi, Aubergine and Argonaute 3 (Ago3) — bind piRNA populations with a length that peaks at 26, 25 and 24 nt, respectively. It is assumed that these size profiles are a signature of a 3′–5′ exonuclease that trims the 3′ ends of

piRNA intermediates that are already loaded into a Piwi protein, and all of the different Piwi paralogues leave a different footprint on the maturing piRNA. Indeed, 3′–5′ exonucleolytic activity has been demonstrated to act on longer RNA molecules loaded into PIWI proteins in cell extracts *in vitro*[63]. The enzyme responsible for this activity has not yet been identified but has been named Trimmer. Coupled with Trimmer activity is the methylation activity of Hen1, which adds a methyl group to the piRNA at the 2′OH position of the 3′ end base[63,73–75], similarly to the plant enzyme HEN1 during miRNA biogenesis[76]. This methylation protects piRNAs from the addition of non-templated uridylation and destabilization[73–75,77]. Such uridylation seems to be a general feature of small RNAs, including miRNAs and small interfering RNAs (siRNAs), whenever (near) perfect base pairing to target RNA molecules is involved[78,79]. Hen1 finalizes piRNA biogenesis and piRNA target silencing can commence.

piRNA target silencing

Although piRNA-independent target recognition by PIWI proteins has been proposed[80], we will focus on piRNA-mediated target silencing. One still largely open question regarding piRNA-mediated target recognition is how strong the interactions between the piRNA and the target RNA have to be to trigger a response. In the case of piRNAs targeting transposons, many perfectly base-pairing target RNAs are produced in the germ line, and this has led to the idea that, in general, perfect base pairing is the rule for piRNA–target RNA interactions. However, in *C. elegans*, it has now been demonstrated that mismatched base-pairing interactions between piRNAs and target RNAs can be sufficient to generate a response as well[81,82]. This result begs for a closer examination of piRNA targets in systems in which targets have not yet been identified, such as for the piRNAs expressed during pachytene stages in mammals[46,48,49]. In this stage of meiosis, the homologous chromosomes completely pair and chromosomal crossover occurs. These pachytene piRNAs are expressed in tremendous quantities and seem to be required for proper germ cell maturation, as male mice that lack their PIWI protein partner MIWI are sterile owing to meiotic defects. Yet for most pachytene piRNA species, no perfectly base-pairing target RNA (other than potential antisense transcripts from pachytene piRNA-encoding loci themselves) is produced by the host genome. Applying the mismatch rules found in *C. elegans* may partly solve this issue of pachytene piRNA target identification, although we note that it has been reported that MIWI-catalysed cleavage requires near complete target base pairing[83] and that *D. melanogaster* Piwi is also quite intolerant to mismatches between piRNA and target RNA[84].

Regardless of the manner of base pairing, target silencing should follow target recognition. Some PIWI proteins silence their targets in the cytoplasm in a post-transcriptional manner. As discussed before, PIWI proteins can cleave their target RNAs[24,29], but effects of PIWI proteins on translation have also been reported[35]. However, it has long been suspected that PIWI proteins may also act transcriptionally because some PIWI protein homologues go into the nucleus after they have been loaded with a piRNA[57,85–89]. Consistent with nuclear PIWI protein localization, the mouse PIWI proteins MILI and MIWI2 together lead to DNA methylation at piRNA target loci[28,86,90]. Unfortunately, more detailed information on nuclear PIWI activity has long been lacking, and hence the well-studied nuclear RNA interference (RNAi) pathway of the fission yeast *Schizosaccharomyces pombe* is often used as a template for nuclear PIWI protein activity. In *S. pombe*, a nuclear RNA-induced transcriptional silencing (RITS) complex is involved in transcriptional silencing processes. RITS contains an Argonaute protein (namely, Ago1, which is loaded with siRNAs) that recognizes nascent pericentromeric transcripts. This binding results in the recruitment of another complex containing the histone methyltransferase Clr4 that can methylate H3K9. This in turn leads to the binding of Swi6, a homologue of HP1, resulting in heterochromatin formation. In addition, a short interfering RNA (siRNA)-amplifying machinery is recruited to these targets, establishing a potent silencing response. For reviews that specifically focus on this topic see, for example, REFS 18,37,38. Despite these ideas, causal links between PIWI protein activity and transcriptional effects have long remained unclear, but several recent studies in both *D. melanogaster* and *C. elegans* now shed more light on nuclear roles for piRNAs.

***PIWI-mediated heterochromatin and germline immortality in* C. elegans.** Recent data from several laboratories have shown that the *C. elegans* PIWI pathway can affect the chromatin structure of target loci[91–93]. Interestingly, PRG-1 (which is the main *C. elegans* PIWI protein) itself does not enter the nucleus. Rather, on PRG-1–target interaction in the cytoplasm, a class of small RNAs (namely, 22G RNAs) is synthesized by RNA-dependent RNA polymerases (RdRPs) using the PRG-1-targeted RNA as a template[39,40]. PRG-1 endonucleolytic activity does not seem to be required for this[39,40]. These 22G RNAs are then loaded into a distinct set of worm-specific Argonaute proteins, one of them being WAGO-9 (also known as HRDE-1). Presumably following loading with a 22G RNA, WAGO-9 goes into the nucleus[92–94]. In the nucleus, WAGO-9, with help of previously identified nuclear RNAi factors such as NRDE-1, NRDE-2 and NRDE-4 (REFS 94–96), triggers transcriptional silencing (FIG. 3a). This is accompanied by trimethylation of H3K9 (H3K9me3) at the genomic loci that encode the target RNAs[91–93]. Related to these findings, the HP1 homologues *hpl-1* and *hpl-2* have been shown to have a role in the silencing, just like the histone methyltransferase enzymes SET-25 and SET-32 (REFS 91–93).

Interestingly, the silenced state thus established can, through some unknown mechanism, be converted into a state that is stably inherited across generations, and at this stage the silencing becomes independent of PRG-1. This phenomenon is called RNA-induced epigenetic silencing (RNAe). Notably, RNAe does not occur on PRG-1 targets by default, as transgenes engineered to

Pachytene
The third stage of meiotic prophase. Homologous chromosomes are tightly held together by the synaptonemal complex, and homologous recombination ('crossing over') begins.

RNA-induced transcriptional silencing complex
(RITS complex). An RNA interference (RNAi) effector complex required for heterochromatin assembly in fission yeast. It targets centromeric transcripts to induce both histone H3 at lysine 9 (H3K9) methylation and small interfering RNA amplification.

22G RNAs
Short RNA molecules in *Caenorhabditis elegans* made by RNA-dependent RNA polymerase (RdRP) activity on an RNA template. 22G RNAs are characterized by a triphosphate group at their 5′ end and a strong preference for a G at their 5′ end. They are bound by Argonaute proteins.

RNA-dependent RNA polymerases
(RdRP). RNA polymerases that use single-stranded RNA as a template to synthesize double-stranded RNA.

RNA-induced epigenetic silencing
(RNAe). A form of epigenetic silencing that can be induced by various forms of RNA interference (RNAi)-related pathways, resulting in trimethylation of histone H3 at lysine 9 (H3K9) residues at the silenced locus. The silencing is maintained through both mitosis and meiosis and can last for tens of generations.

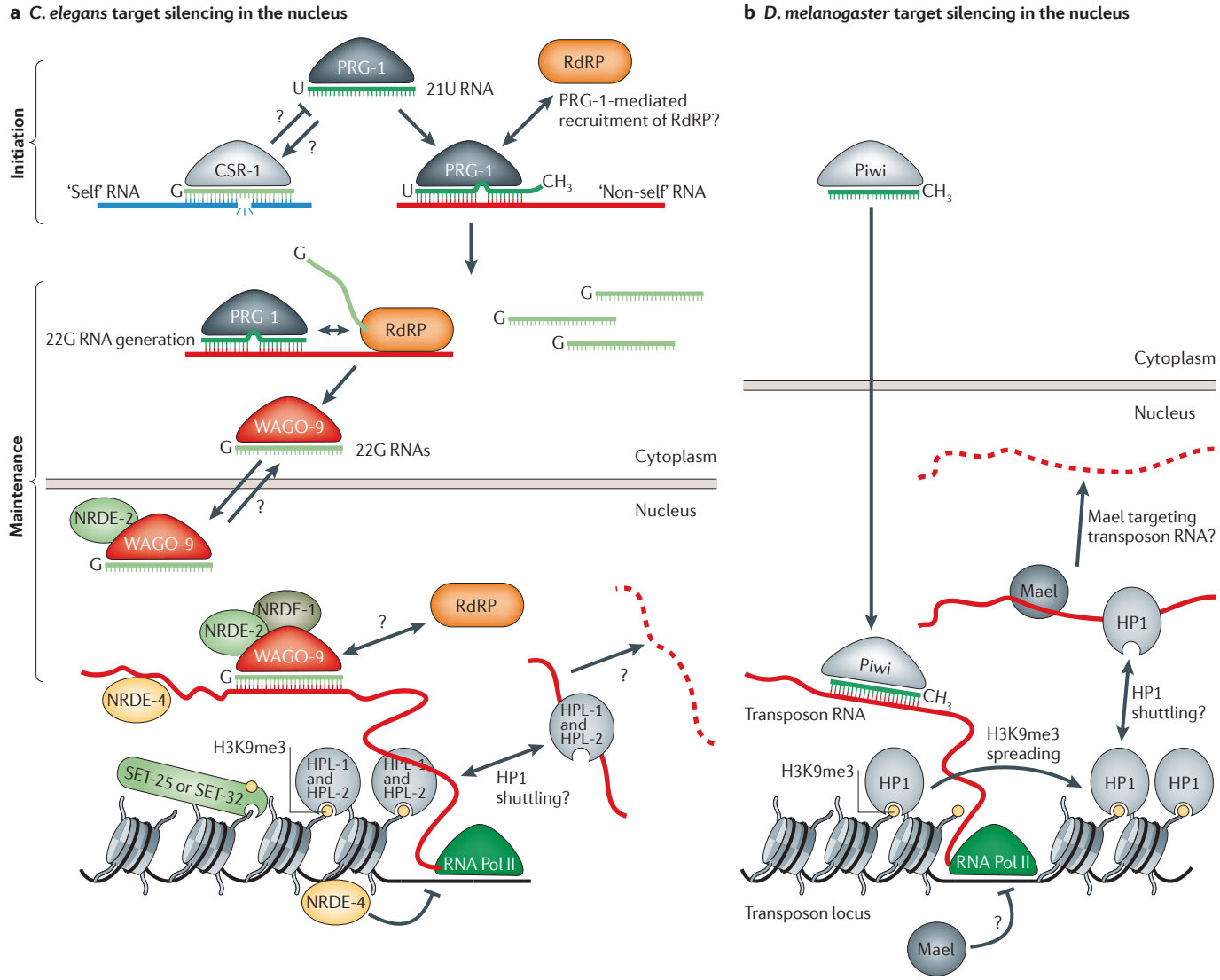

Figure 3 | **Effects of PIWI pathways on chromatin.** **a** | In *Caenorhabditis elegans*, the PIWI protein PRG-1 triggers an RNA-dependent RNA polymerase (RdRP) enzyme to produce 22G RNAs using PRG-1-targeted RNA molecules in the cytoplasm as a template[81,82]. The Argonaute protein WAGO-9 accepts these 22G RNAs and shuttles into the nucleus, where it triggers transcriptional gene silencing, resulting in trimethylation of histone H3 at lysine 9 (H3K9) residues by histone methyltransferases[91–94]. Two such methylases have been identified — SET-25 and SET-32 (REFS 92,93) — but their specificities still have to be determined. Consistently, heterochromatin protein 1 (HP1)-like proteins HPL-1 and HPL-2 have been shown to be required for the silencing effects. Analogously to the nuclear Argonaute protein NRDE-3, it is presumed that WAGO-9 interacts with target loci via nascent RNA transcripts and leads to inhibition of RNA polymerase II (RNA Pol II) elongation via NRDE-4 (REFS 94,96). We show the shuttling of HPL-1 and HPL-2 between chromatin and RNA-bound states, combined with RNA destabilization in analogy to the work from Keller *et al.*[52] in *Schizosaccharomyces pombe*. Through an unknown mechanism, the nuclear silencing can become independent of PRG-1. Hence, the steps including PRG-1 are referred to as initiation steps, whereas further downstream, steps are considered to be maintenance steps. We note that this maintenance thus involves the generation of 22G RNAs in the absence of PRG-1 and thus may involve a novel mechanism through which RdRP enzymes are recruited to (possibly nascent) transcripts. The CSR-1 pathway is depicted downstream of PRG-1, reflecting the idea that CSR-1 may be responsible for the observed capacity of some loci to overrule PRG-1-mediated silencing, perhaps by cleaving PRG-1 targets to prevent RdRP activity. However, direct experimental support for these steps is lacking. **b** | In *Drosophila melanogaster*, Piwi has been shown to be responsible for H3K9 trimethylation of euchromatic transposon copies[84,104–106]. Although it has been suggested that this interaction may indeed proceed via nascent transcript[84], this still needs to be further resolved. HP1 then binds these H3K9me3 marks. Following Piwi targeting, RNA Pol II occupancy at promoters is reduced, suggesting that this pathway affects initiation of transcription. However, more molecular detail is required to make strong statements. Maelstrom (Mael) is one of the factors that mediates the effects of Piwi-induced silencing downstream of H3K9 trimethylation. Its exact roles are, however, unclear. We depict two possibilities that may both occur: direct inhibition of some step during RNA Pol II transcription and interaction with transcripts coming from silenced loci. Again, we depict hypothetical shuttling of HP1 proteins.

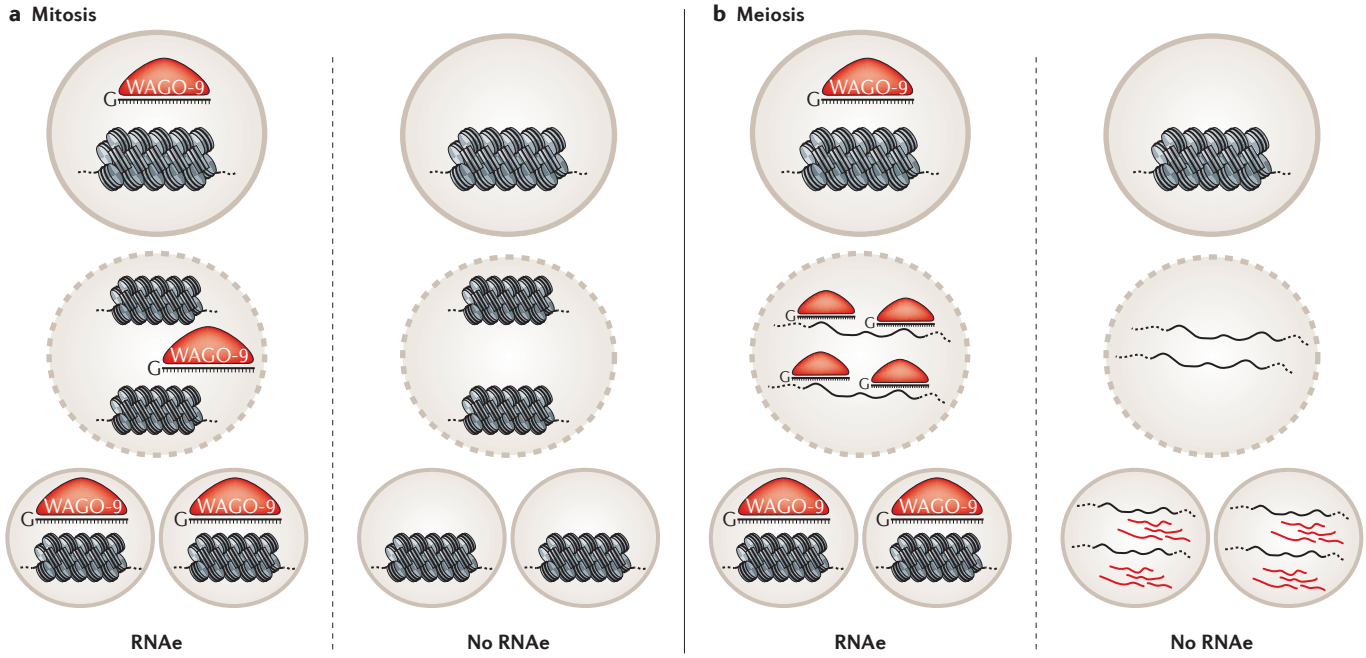

Figure 4 | **Transgenerational inheritance of PIWI-induced heterochromatin.** **a** | Mitotic inheritance of a PRG-1-mediated heterochromatic state (represented as the condensed structure flanked by other genomic DNA, which is represented as a dashed line) in *Caenorhabditis elegans*. Presumably, the epigenetic state of the locus is not reset during mitosis, and hence no RNA-induced epigenetic silencing (RNAe) pathway is required to maintain the heterochromatic state. This mode probably operates during mitotic germline development within an individual. **b** | A meiotic division during which the epigenetic state of a PRG-1-targeted locus may be reset (represented by the change of the condensed structure to an open, linear structure of the locus; note that this has not been directly demonstrated). To re-establish the heterochromatic state after meiosis, RNAe, which involves WAGO-9 bound with 22G RNAs that are homologous to the locus that needs to be re-silenced, is required. The situation may be similar in mice in which PIWI proteins have been shown to be required to re-establish DNA methylation at PIWI target loci.

be targeted by PRG-1 can maintain their dependence on PRG-1 for continued silencing in wild-type animals[81,91]. The distinction and transition between the PRG-1-dependent and PRG-1-independent silent states are currently unclear.

Mutational analysis of this transgenerational silencing effect has indicated that the transmission of the silenced state through meiosis requires WAGO-9, the NRDE factors and other RNAi-related factors, such as MUT-7 and RDE-3 (REFS 91–93). Interestingly, WAGO-9 mutants display an additional phenotype: their germ cells lose their characteristic immortality, such that after several generations, WAGO-9 mutants become sterile. This is accompanied by loss of H3K9me3 marks at target loci[94,95]. The molecular reasons behind this phenotype are not clear, but it suggests a requirement in *C. elegans* for PRG-1 to act through WAGO-9 to stably maintain proper gene expression patterns in the germ cells over generations. Although PRG-1 may initiate fairly stable forms of gene silencing, the stability is probably less than 100%, leading to sporadic activation of germline-incompatible genes. If they are not re-silenced through RNAe, such events may eventually mount up to sterility. Whether similar effects are at play in other systems is hard to judge, given the immediate sterility of PIWI mutants in most other animals studied[1,2,22,97].

Although transgenerational silencing — for example, maintenance of silencing through meiosis — requires the various RNAi-related factors mentioned above, the maintenance of silencing through mitosis within one individual does not depend on these factors[91–93]. How can this difference be explained? Possibly, mitosis does not require RNAi to maintain established RNAe-related heterochromatin, just as other types of chromatin are maintained independently of RNAi. By contrast, inheritance through meiosis may require RNAi to store the silencing information as, at least in mammals, meiosis involves an epigenetic resetting process[98]. The information stored in the form of small RNAs would allow the re-establishment of heterochromatin after meiosis has finished (FIG. 4). Such a system would closely mirror the situation in mouse germ cells, in which it has been proposed that the effect of *Mili* and *Miwi2* mutations on the remethylation of certain transposons reflects such a storage of information on transposon silencing as well[28,86,90].

Finally, several observations suggest the existence of a pathway that counteracts PRG-1-induced silencing in *C. elegans*. These observations include the capability of certain transgenes to re-activate RNAe-silenced transgenes dominantly. It has been suggested that another RNAi-related pathway mediated by the Argonaute protein CSR-1 may be responsible for this[92,93].

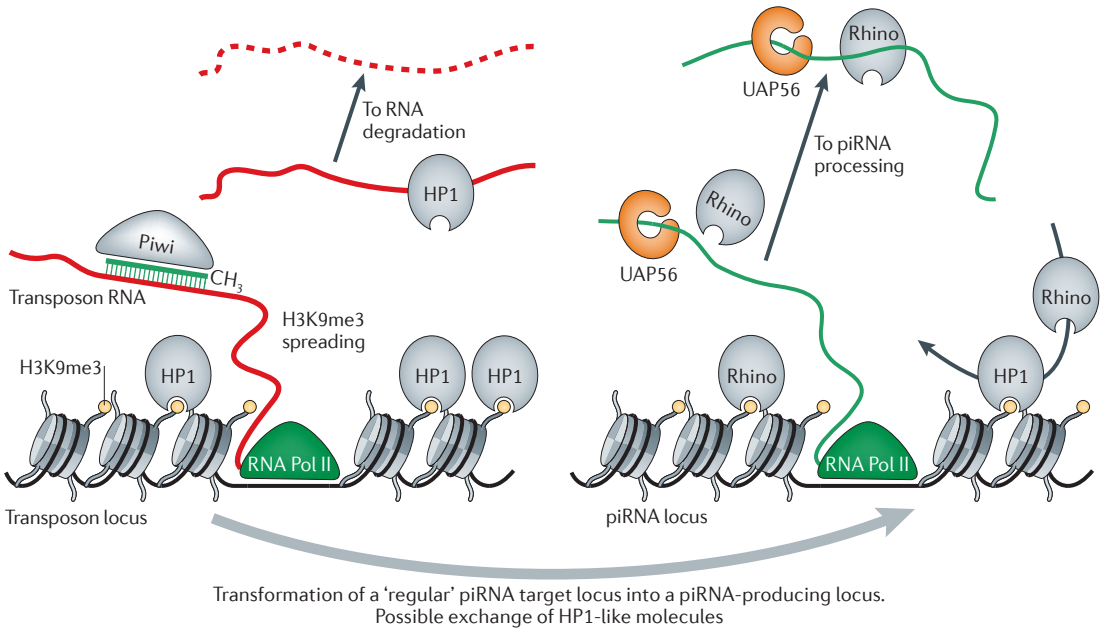

Figure 5 | **Conversion of a Piwi target locus into a piRNA-producing locus.** In *Drosophila melanogaster*, a Piwi-interacting RNA (piRNA) target locus can be turned into a piRNA-producing locus[109]. Although the inheritance of this trait seems to be largely cytoplasmic, associated chromatin changes may still be at the basis of their conversion into piRNA-producing loci. What is depicted here is a purely hypothetical process in which 'regular' heterochromatin protein 1 (HP1) proteins that silence the Piwi target are replaced by Rhino-like HP1 molecules, channelling the transcripts into piRNA processing rather than to destine them for destruction. Clearly, substantial experimentation is needed to test this model in the various model systems.

This idea is based on the fact that CSR-1 also acts in the germ line and binds 22G RNAs derived from genes expressed in the germ line[99,100] and that loss of CSR-1 does not activate its targets. As CSR-1, like WAGO-9, binds 22G RNAs, there must be a factor that initiates RdRP activity on CSR-1 target RNAs. It is worthwhile to consider that PRG-1 may be this factor, just as PRG-1 drives WAGO-9 22G RNA production. In such a scenario, PRG-1 may in some way set a balance between silencing and activating pathways by providing a proper assessment of the 'selfness' of a given sequence. Obviously, these ideas need to be tested, but they represent an interesting line of thought. Curiously, a recent report indicates that the unicellular organism *Oxytrichia trifallax* — which undergoes extensive deletion of genomic sequences as part of its life cycle — uses its Piwi pathway to protect particular sequences from being deleted[101], and also *D. melanogaster* Piwi has at least once been implicated in activation of target loci[84,102].

***Piwi-mediated heterochromatin formation in* D. melanogaster.** Although the Piwi proteins Aub and Ago3 are cytoplasmic, Piwi itself is nuclear. In fact, direct interactions between Piwi and heterochromatin factors such as HP1 have been described[103]. However, whether Piwi affects chromatin has long been unclear. A number of recent studies shed more light on a nuclear role for Piwi in transcriptional gene silencing[84,104–106]. The general picture that emerges is that loss of Piwi leads to a strong reduction of histone H3 trimethylated at lysine 9 (H3K9me3) marks on Piwi targets in euchromatic regions. This is accompanied by an increase in RNA polymerase II (RNA Pol II) occupancy at the promoters of Piwi targets and an increase in nascent RNA transcript levels at target loci. Most probably, these effects do not require Piwi cleavage of the target RNA[107]. Despite this inverse correlation between Piwi presence and chromatin immunoprecipitation (ChIP) signals for RNA Pol II occupancy, colocalization of Piwi and RNA Pol II on nurse-cell-derived polytene chromosomes has been demonstrated[106]. Although the resolution of such studies is low, it suggests that Piwi target loci in wild-type cells still attract RNA Pol II. Interesting light has been shed on the silencing mechanism through analysing Maelstrom (Mael) mutants[105]. Loss of Mael, which is another known player in the Piwi pathway[108], results in transposon activation, although piRNA levels and Piwi loading are largely normal. Intriguingly, although transposons are activated in Mael mutants, H3K9me3 levels at these loci were almost unchanged, indicating that H3K9me3 marks are by themselves not sufficient to silence gene expression and that Mael functions downstream of Piwi and H3K9 trimethylation steps (FIG. 4b). Intriguingly, in Mael mutants, RNA Pol II occupancy was increased on Piwi target loci, and the H3K9me3 mark spread to regions downstream of the original target. The authors suggest a model in which the spreading of H3K9me3 marks in Mael mutants may in fact be a consequence of the increase in RNA Pol II transcription, as an increase of nascent transcripts at target loci may recruit more

Paramutation
The interaction between two alleles through which the epigenetic state of one allele can be imposed on the other allele.

Piwi proteins to that locus. In agreement with this notion, RNA Pol II transcription of individual transposon insertion loci was indeed found to be required for Piwi-mediated silencing and heterochromatinization[105]. The inheritance of the Piwi-mediated heterochromatin chromatin has not been directly studied yet in *D. melanogaster*, but given the absence of secondary RNAi pathways coupling to Piwi, as is the case in *C. elegans*, it seems likely that *D. melanogaster* Piwi will be required at each generation to maintain proper heterochromatin at Piwi targets.

Finally, it was found that Piwi-induced silencing has an impact on genes that are physically close to targeted transposons[105]. Apparently, the effects of the chromatin marks established by the Piwi pathway are not restricted to the primary targets but can extend to sequences that are close-by. Thus, this pathway may affect the transcription of endogenous genes without directly targeting them. It is perhaps because of this effect that the Piwi pathway, at least in some organisms, may also act to sustain the expression of essential genes that may otherwise be potentially silenced (see above).

Concluding remarks

Going full circle: piRNAs from targeted loci? From the advances described in the above sections, it has become clear that both PIWI target silencing and piRNA precursor specification depend on similar types of chromatin that are characterized by H3K9me3 marks and HP1-like proteins. Intriguingly, there is now evidence that the silencing activity of a Piwi pathway can turn a target locus into a piRNA-generating locus. A recent study has revealed that in *D. melanogaster*, a non-piRNA-producing locus (such as a protein-coding gene) can be turned into a piRNA-producing one simply by bringing it into contact with homologous piRNAs[109]. During and after this process, the locus continues to be transcribed, but rather than leading to protein expression, the transcripts are now processed into piRNAs. Effectively, this silences the gene and re-routes the transcripts into the piRNA pathway. The resulting piRNAs can in turn silence, in *trans*, other loci of similar sequence[109]. As the effect can be maintained over generations as long as the involved Piwi pathway is intact, the authors compare their observations to paramutation, which is a phenomenon that entails the change of protein-expressing loci into silent loci that can in turn silence other homologous loci[110,111]. In this intriguing case of *D. melanogaster* paramutation[109], it remains to be tested to what extent the chromatin structure of the transformed locus changes and whether it starts to recruit proteins such as Rhino and UAP56 that seem to be involved in trafficking precursor piRNA transcripts (see above). Still, already at this point in time, the remarkable similarities between the chromatin state that is required at a piRNA-producing locus and the chromatin state resulting from the transcriptional silencing of a target locus by PIWI proteins are striking. Everything considered, the findings may fit a model in which piRNAs are amplified not only post-transcriptionally — through ping-pong amplification or RdRP activities — but perhaps also transcriptionally by converting piRNA target loci into piRNA-producing loci (FIG. 5). Clearly, these ideas are in dire need of more experimental support, and most probably the situation will be more complex than sketched here. We may even find that in different species, different strategies have been adopted to specify piRNA transcripts and/or to silence Piwi protein targets and that the generalizing statements made here are far too simple. Screens such as those recently published for *D. melanogaster*, which are aimed at the identification of factors involved in the various steps of the Piwi pathway, will be of big help in this respect[112–114]. We are very much looking forward to the studies that will contribute to solving the many questions that remain in this field.

1. Cox, D. N. *et al.* A novel class of evolutionarily conserved genes defined by PIWI are essential for stem cell self-renewal. *Genes Dev.* **12**, 3715–3727 (1998).
2. Deng, W. & Lin, H. *Miwi*, a murine homolog of *Piwi*, encodes a cytoplasmic protein essential for spermatogenesis. *Dev. Cell* **2**, 819–830 (2002).
3. Ghildiyal, M. & Zamore, P. D. Small silencing RNAs: an expanding universe. *Nature Rev. Genet.* **10**, 94–108 (2009).
4. Song, J. J. Crystal structure of argonaute and its implications for RISC slicer activity. *Science* **305**, 1434–1437 (2004).
5. Tolia, N. H. & Joshua-Tor, L. Slicer and the Argonautes. *Nature Chem. Biol.* **3**, 36–43 (2007).
6. Wang, Y. *et al.* Nucleation, propagation and cleavage of target RNAs in Ago silencing complexes. *Nature* **461**, 754–761 (2009).
7. Schirle, N. T. & MacRae, I. J. The crystal structure of human Argonaute2. *Science* **336**, 1037–1040 (2012).
8. Elkayam, E. *et al.* The structure of human argonaute-2 in complex with miR-20a. *Cell* **150**, 100–110 (2012).
9. Wang, Y. *et al.* Structure of an Argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. *Nature* **456**, 921–926 (2008).
10. van Wolfswinkel, J. C. & Ketting, R. F. The role of small non-coding RNAs in genome stability and chromatin organization. *J. Cell Sci.* **123**, 1825–1839 (2010).
11. Czech, B. & Hannon, G. J. Small RNA sorting: matchmaking for Argonautes. *Nature Rev. Genet.* **12**, 19–31 (2010).
12. Siomi, H. & Siomi, M. C. On the road to reading the RNA-interference code. *Nature* **457**, 396–404 (2009).
13. Jinek, M. & Doudna, J. A. A three-dimensional view of the molecular machinery of RNA interference. *Nature* **457**, 405–412 (2009).
14. Rana, T. M. Illuminating the silence: understanding the structure and function of small RNAs. *Nature Rev. Mol. Cell Biol.* **8**, 23–36 (2007).
15. Malone, C. D. & Hannon, G. J. Small RNAs as guardians of the genome. *Cell* **136**, 656–668 (2009).
16. Kazazian, H. H. Mobile elements: drivers of genome evolution. *Science* **303**, 1626–1632 (2004).
17. Rouget, C. *et al.* Maternal mRNA deadenylation and decay by the piRNA pathway in the early *Drosophila* embryo. *Nature* **467**, 1128–1132 (2010).
18. Castel, S. E. & Martienssen, R. A. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. *Nature Rev. Genet.* **14**, 100–112 (2013).
19. Robine, N. *et al.* A broadly conserved pathway generates 3′UTR-directed primary piRNAs. *Curr. Biol.* **19**, 2066–2076 (2009).
20. Seto, A. G., Kingston, R. E. & Lau, N. C. The coming of age for PIWI proteins. *Mol. Cell* **26**, 603–609 (2007).
21. Rajasethupathy, P. *et al.* A role for neuronal piRNAs in the epigenetic control of memory-related synaptic plasticity. *Cell* **149**, 693–707 (2012).
22. Houwing, S. *et al.* A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell* **129**, 69–82 (2007).
23. Vagin, V. V. A. Distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**, 320–324 (2006).
24. Brennecke, J. *et al.* Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* **128**, 1089–1103 (2007).
25. Li, X. Z. *et al.* An ancient transcription factor initiates the burst of piRNA production during early meiosis in mouse testes. *Mol. Cell* **50**, 67–81 (2013).
This paper describes the first transcription factor that initiates the transcription of pachytene piRNA precursors and genes involved in piRNA biogenesis, thereby creating a feed-forward loop to boost piRNA abundance.
26. Al-Muktar, K. A. K. & Webb, A. C. An ultrastructural study of primordial germ cells, oogonia and early oocytes in *Xenopus laevis*. *J. Embryol. Exp. Morphol.* **26**, 195–217 (1971).
27. Ketting, R. F. The many faces of RNAi. *Dev. Cell* **20**, 148–161 (2011).
28. Aravin, A. A., Sachidanandam, R., Girard, A., Fejes-Toth, K. & Hannon, G. J. Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science* **316**, 744–747 (2007).
29. Gunawardane, L. S. *et al.* A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in *Drosophila*. *Science* **315**, 1587–1590 (2007).
30. Li, C. *et al.* Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* **137**, 509–521 (2009).

31. Grentzinger, T. *et al.* piRNA-mediated transgenerational inheritance of an acquired trait. *Genome Res.* **22**, 1877–1888 (2012).
32. Brennecke, J. *et al.* An epigenetic role for maternally inherited piRNAs in transposon silencing. *Science* **322**, 1387–1392 (2008).
33. Klattenhoff, C. & Theurkauf, W. Biogenesis and germline functions of piRNAs. *Development* **135**, 3–9 (2007).
34. Siomi, M. C., Sato, K., Pezic, D. & Aravin, A. A. PIWI-interacting small RNAs: the vanguard of genome defence. *Nature Rev. Mol. Cell Biol.* **12**, 246–258 (2011).
35. Grivna, S. T., Pyhtila, B. & Lin, H. MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc. Natl Acad. Sci. USA* **103**, 13415–13420 (2006).
36. Molnar, A., Melnyk, C. & Baulcombe, D. C. Silencing signals in plants: a long journey for small RNAs. *Genome Biol.* **12**, 215 (2011).
37. hler, M. B. U. & Gasser, S. M. Silent chromatin at the middle and ends: lessons from yeasts. *EMBO J.* **28**, 2149–2161 (2009).
38. Grewal, S. I. RNAi-dependent formation of heterochromatin and its diverse functions. *Curr. Opin. Genet. Dev.* **20**, 134–141 (2010).
39. Das, P. P. *et al.* Piwi and piRNAs act upstream of an endogenous siRNA pathway to suppress Tc3 transposon mobility in the *Caenorhabditis elegans* germline. *Mol. Cell* **31**, 79–90 (2008).
40. Batista, P. J. *et al.* PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in *C. elegans*. *Mol. Cell* **31**, 67–78 (2008).
41. Billi, A. C. *et al.* A conserved upstream motif orchestrates autonomous, germline-enriched expression of *Caenorhabditis elegans* piRNAs. *PLoS Genet.* **9**, e1003392 (2013).
42. Ruby, J. G. *et al.* Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in *C. elegans*. *Cell* **127**, 1193–1207 (2006).
43. Cecere, G., Zheng, G. X. Y., Mansisidor, A. R., Klymko, K. E. & Grishok, A. Promoters recognized by forkhead proteins exist for individual 21U-RNAs. *Mol. Cell* **47**, 734–745 (2012).
44. Gu, W. *et al.* CapSeq and CIP-TAP identify Pol II start sites and reveal capped small RNAs as *C. elegans* piRNA precursors. *Cell* **151**, 1488–1500 (2012).
45. Brannan, K. *et al.* mRNA decapping factors and the exonuclease Xrn2 function in widespread premature termination of RNA polymerase II transcription. *Mol. Cell* **46**, 311–324 (2012).
46. Aravin, A. *et al.* A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* **442**, 203–207 (2006).
47. Aravin, A. A., Hannon, G. J. & Brennecke, J. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* **318**, 761–764 (2007).
48. Lau, N. C. *et al.* Characterization of the piRNA complex from rat testes. *Science* **313**, 363–367 (2006).
49. Girard, A., Sachidanandam, R., Hannon, G. J. & Carmell, M. A. A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* **442**, 199–202 (2006).
50. Klattenhoff, C. *et al.* The *Drosophila* HP1 homolog rhino is required for transposon silencing and piRNA production by dual-strand clusters. *Cell* **138**, 1137–1149 (2009).
51. Grewal, S. I. S. & Jia, S. Heterochromatin revisited. *Nature Rev. Genet.* **8**, 35–46 (2007).
52. Keller, C. *et al.* HP1(Swi6) mediates the recognition and destruction of heterochromatic RNA transcripts. *Mol. Cell* **47**, 215–227 (2012).
This work shows the RNA-binding activity of SWI6 is required for silencing but leaves heterochromatin formation intact.
53. Zhang, F. *et al.* UAP56 couples piRNA clusters to the perinuclear transposon silencing machinery. *Cell* **151**, 871–884 (2012).
This paper describes the roles of nuclear UAP56 and perinuclear Vasa proteins in forming a piRNA-processing compartment that spans the nuclear envelope and is essential for piRNA production and transposon silencing.
54. Pane, A. Wehr, K. & Schüpbach, T. Zucchini and squash encode two putative nucleases required for rasiRNA production in the *Drosophila* germline. *Dev. Cell* **12**, 851–862 (2007).
55. Huang, H. *et al.* piRNA-associated germline nuage formation and spermatogenesis require MitoPLD profusogenic mitochondrial-surface lipid signaling. *Dev. Cell* **20**, 376–387 (2011).
56. Watanabe, T. *et al.* MITOPLD is a mitochondrial protein essential for nuage formation and piRNA biogenesis in the mouse germline. *Dev. Cell* **20**, 364–375 (2011).
57. Saito, K. *et al.* Roles for the Yb body components Armitage and Yb in primary piRNA biogenesis in *Drosophila*. *Genes Dev.* **24**, 2493–2498 (2010).
58. Choi, S.-Y. *et al.* A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. *Nature Cell Biol.* **8**, 1255–1262 (2006).
59. Voigt, F. *et al.* Crystal structure of the primary piRNA biogenesis factor Zucchini reveals similarity to the bacterial PLD endonuclease Nuc. *RNA* **18**, 2128–2134 (2012).
60. Nishimasu, H. *et al.* Structure and function of Zucchini endoribonuclease in piRNA biogenesis. *Nature* **491**, 284–287 (2012).
61. Ipsaro, J. J., Haase, A. D., Knott, S. R., Joshua-Tor, L. & Hannon, G. J. The structural biochemistry of Zucchini implicates it as a nuclease in piRNA biogenesis. *Nature* **491**, 279–283 (2012).
References 59, 60 and 61 show crystal structures of Zuc, showing that it has a nuclease domain and possesses single-stranded nuclease activity. These studies provide proof that Zuc is a nuclease that probably acts to specify piRNA 5′ ends.
62. Mi, S. *et al.* Sorting of small RNAs into *Arabidopsis* Argonaute complexes is directed by the 5′ terminal nucleotide. *Cell* **133**, 116–127 (2008).
63. Kawaoka, S., Izumi, N., Katsuma, S. & Tomari, Y. 3′ end formation of PIWI-interacting RNAs *in vitro*. *Mol. Cell* **43**, 1015–1022 (2011).
64. Iwasaki, S. *et al.* Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol. Cell* **39**, 292–299 (2010).
65. Johnston, M., Geoffroy, M.-C., Sobala, A., Hay, R. & Hutvagner, G. HSP90 protein stabilizes unloaded Argonaute complexes and microscopic P-bodies in human cells. *Mol. Biol. Cell* **21**, 1462–1469 (2010).
66. Miyoshi, T., Takeuchi, A., Siomi, H. & Siomi, M. C. A direct role for Hsp90 in pre-RISC formation in *Drosophila*. *Nature Struct. Mol. Biol.* **17**, 1024–1026 (2010).
67. Iki, T., Yoshikawa, M., Meshi, T. & Ishikawa, M. Cyclophilin 40 facilitates HSP90-mediated RISC assembly in plants. *EMBO J.* **31**, 267–278 (2012).
68. Gangaraju, V. K. *et al. Drosophila* Piwi functions in Hsp90-mediated suppression of phenotypic variation. *Nature Genet.* **43**, 153–158 (2010).
69. Olivieri, D., Senti, K.-A., Subramanian, S., Sachidanandam, R. & Brennecke, J. The cochaperone shutdown defines a group of biogenesis factors essential for all piRNA populations in *Drosophila*. *Mol. Cell* **47**, 954–969 (2012).
70. Specchia, V. *et al.* Hsp90 prevents phenotypic variation by suppressing the mutagenic activity of transposons. *Nature* **463**, 662–665 (2010).
71. Vagin, V. V. *et al.* Proteomic analysis of murine Piwi proteins reveals a role for arginine methylation in specifying interaction with Tudor family members. *Genes Dev.* **23**, 1749–1762 (2009).
72. Xiol, J. *et al.* A role for Fkbp6 and the chaperone machinery in piRNA amplification and transposon silencing. *Mol. Cell* **47**, 970–979 (2012).
73. Kamminga, L. M. *et al.* Hen1 is required for oocyte development and piRNA stability in zebrafish. *EMBO J.* **29**, 3688–3700 (2010).
74. Saito, K. *et al.* Pimet, the *Drosophila* homolog of HEN1, mediates 2′-O-methylation of Piwi-interacting RNAs at their 3′ ends. *Genes Dev.* **21**, 1603–1608 (2007).
75. Horwich, M. D. *et al.* The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Curr. Biol.* **17**, 1265–1272 (2007).
76. Yu, B. Methylation as a crucial step in plant microRNA biogenesis. *Science* **307**, 932–935 (2005).
77. Kurth, H. M. & Mochizuki, K. 2′-O-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in *Tetrahymena*. *RNA* **15**, 675–685 (2009).
78. Ameres, S. L. *et al.* Target RNA-directed trimming and tailing of small silencing RNAs. *Science* **328**, 1534–1539 (2010).
79. Ameres, S. L., Hung, J.-H., Xu, J., Weng, Z. & Zamore, P. D. Target RNA-directed tailing and trimming purifies the sorting of endo-siRNAs between the two *Drosophila* Argonaute proteins. *RNA* **17**, 54–63 (2011).
80. Vourekas, A. *et al.* Mili and Miwi target RNA repertoire reveals piRNA biogenesis and function of Miwi in spermiogenesis. *Nature Struct. Mol. Biol.* **19**, 773–781 (2012).
81. Bagijn, M. P. *et al.* Function, targets, and evolution of *Caenorhabditis elegans* piRNAs. *Science* **337**, 574–578 (2012).
82. Lee, H.-C. *et al. C. elegans* piRNAs mediate the genome-wide surveillance of germline *Transcripts*. *Cell* **150**, 78–87 (2012).
References 81 and 82 show that the *C. elegans*-specific PIWI protein PRG-1 binds its targets by imperfectly base pairing and silences independently of its endonuclease activity. Instead, it recruits a downstream small RNA pathway that generates endogenous siRNAs (endo-siRNAs) to induce silencing.
83. Reuter, M. *et al.* Miwi catalysis is required for piRNA amplification-independent LINE1 transposon silencing. *Nature* **480**, 264–267 (2011).
84. Huang, X. A. *et al.* A major epigenetic programming mechanism guided by piRNAs. *Dev. Cell* **24**, 502–516 (2013).
85. Cox, D. N., Chao, A. & Lin, H. piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503–514 (2000).
86. Aravin, A. A. *et al.* A piRNA pathway primed by individual transposons is linked to *de novo* DNA methylation in mice. *Mol. Cell* **31**, 785–799 (2008).
87. Houwing, S., Berezikov, E. & Ketting, R. F. Zili is required for germ cell differentiation and meiosis in zebrafish. *EMBO J.* **27**, 2702–2711 (2008).
88. Qi, H. *et al.* The Yb body a major site for Piwi-associated RNA biogenesis and a gateway for Piwi expression and transport to the nucleus in somatic cells. *J. Biol. Chem.* **286**, 3789–3797 (2011).
89. Olivieri, D., Sykora, M. M., Sachidanandam, R., Mechtler, K. & Brennecke, J. An *in vivo* RNAi assay identifies major genetic and cellular requirements for primary piRNA biogenesis in *Drosophila*. *EMBO J.* **29**, 3301–3317 (2010).
90. Kuramochi-Miyagawa, S. *et al.* MVH in piRNA processing and gene silencing of retrotransposons. *Genes Dev.* **24**, 887–892 (2010).
91. Luteijn, M. J. *et al.* Extremely stable Piwi-induced gene silencing in *Caenorhabditis elegans*. *EMBO J.* **31**, 3422–3430 (2012).
92. Ashe, A. *et al.* piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88–99 (2012).
93. Shirayama, M. *et al.* piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* **150**, 65–77 (2012).
94. Buckley, B. A. *et al.* A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* **489**, 447–451 (2012).
References 91, 92, 93 and 94 show a role for PRG-1 in inducing stably inherited silenced state by recruiting a nuclear RNAi machinery that triggers heterochromatin formation. Reference 94 demonstrates the nuclear activity of WAGO-9 in RNAi-induced transgenerational silencing and reveals a role in germline mortality.
95. Burton, N. O., Burkhart, K. B. & Kennedy, S. Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci.* **108**, 19683–19688 (2011).
96. Burkhart, K. B. *et al.* A pre-mRNA-associating factor links endogenous siRNAs to chromatin regulation. *PLoS Genet.* **7**, e1002249 (2011).
97. Kuramochi-Miyagawa, S. *et al.* Mili, a mammalian member of Piwi family gene, is essential for spermatogenesis. *Development* **131**, 839–849 (2004).
98. Morgan, H. D., Santos, F., Green, K., Dean, W. & Reik, W. Epigenetic reprogramming in mammals. *Hum. Mol. Genet.* **14** (Suppl. 1), R47–R58 (2005).
99. Claycomb, J. M. *et al.* The Argonaute CSR-1 and its 22G-RNA cofactors are required for holocentric chromosome segregation. *Cell* **139**, 123–134 (2009).
100. Wolfswinkel, J. C. V. *et al.* CDE-1 affects chromosome segregation through uridylation of CSR-1-bound siRNAs. *Cell* **139**, 135–148 (2009).

101. Fang, W., Wang, X., Bracht, J. R., Nowacki, M. & Landweber, L. F. Piwi-interacting RNAs protect DNA against loss during oxytricha genome rearrangement. *Cell* **151**, 1243–1255 (2012).
102. Yin, H. & Lin, H. An epigenetic activation role of Piwi and a Piwi-associated piRNA in *Drosophila melanogaster*. *Nature* **450**, 304–308 (2007).
103. Brower-Toland, B. *et al.* *Drosophila* PIWI associates with chromatin and interacts directly with HP1a. *Genes Dev.* **21**, 2300–2311 (2007).
104. Rozhkov, N. V., Hammell, M. & Hannon, G. J. Multiple roles for Piwi in silencing *Drosophila* transposons. *Genes Dev.* **27**, 400–412 (2013).
105. Sienski, G., Donertas, D. & Brennecke, J. Transcriptional silencing of transposons by Piwi and maelstrom and its impact on chromatin state and gene expression. *Cell* **151**, 964–980 (2012).
106. Le Thomas, A. *et al.* Piwi induces piRNA-guided transcriptional silencing and establishment of a repressive chromatin state. *Genes Dev.* **27**, 390–399 (2013).
References 104, 105 and 106, along with reference 82, show that *D. melanogaster* Piwi is required to establish heterochromatic H3K9me3 marks on transposons and their genomic surroundings.
Reference 105 also shows that the high-mobility group (HMG) protein Mael is essential for transposon silencing but not for heterochromatin formation.
107. Darricarrère, N., Liu, N., Watanabe, T. & Lin, H. Function of Piwi, a nuclear Piwi/Argonaute protein, is independent of its slicer activity. *Proc. Natl Acad. Sci.* **110**, 1297–1302 (2013).
108. Aravin, A. A. *et al.* Cytoplasmic compartmentalization of the fetal piRNA pathway in mice. *PLoS Genet.* **5**, e1000764 (2009).
109. de Vanssay, A. *et al.* Paramutation in *Drosophila* linked to emergence of a piRNA-producing locus. *Nature* **490**, 112–115 (2012).
This work shows that piRNA targets can be converted into piRNA-producing loci, resulting in genetic behavior that closely mirrors that of paramutation.
110. Erhard, K. F. Jr & Hollick, J. B. Paramutation: a process for acquiring *trans*-generational regulatory states. *Curr. Opin. Plant Biol.* **14**, 210–216 (2011).
111. Pilu, R. Paramutation: just a curiosity or fine tuning of gene expression in the next generation? *Curr. Genom.* **12**, 298–306 (2011).
112. Muerdter, F. *et al.* A genome-wide RNAi screen draws a genetic framework for transposon control and primary piRNA biogenesis in *Drosophila*. *Mol. Cell.* http://dx.doi.org/10.1016/j.molcel.2013.04.006 (2013).
113. Czech, B., Preall, J. B., McGinn, J. & Hannon, G. J. A transcriptome-wide RNAi screen in the *Drosophila* ovary reveals factors of the germline piRNA pathway. *Mol. Cell* http://dx.doi.org/10.1016/j.molcel.2013.04.007 (2013).
114. Handler, D. *et al.* The genetic makeup of the *Drosophila* piRNA pathway. *Mol. Cell* http://dx.doi.org/10.1016/j.molcel.2013.04.031 (2013).

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Competing interests statement
The authors declare no competing financial interests.