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REVIEW

piRNAs: from biogenesis to function

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ABSTRACT

Distinguishing self from non-self plays a crucial role in safeguarding the germlines of metazoa from mobile DNA elements. Since their discovery less than a decade ago, Piwi-interacting RNAs (piRNAs) have been shown to repress transposable elements in the germline and, hence, have been at the forefront of research aimed at understanding the mechanisms that maintain germline integrity. More recently, roles for piRNAs in gene regulation have emerged. In this Review, we highlight recent advances made in understanding piRNA function, highlighting the divergent nature of piRNA biogenesis in different organisms, and discussing the mechanisms of piRNA action during transcriptional regulation and in transgenerational epigenetic inheritance.

KEY WORDS: Piwi, piRNA biogenesis, piRNA-mediated transcriptional repression, piRNAs, Transgenerational epigenetic inheritance

Introduction

The discovery of small non-coding RNAs, including microRNAs (miRNAs) and short interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999; Lee et al., 1993; Reinhart et al., 2000) revolutionised our understanding of how gene expression is regulated. These non-coding RNAs are not translated into proteins but instead act through complementary base pairing with target RNAs. Together with their protein partners, the Argonaute proteins, short RNAs form the RNA-induced silencing complexes (RISCs): in the case of miRNAs, for example, this complex is referred to as miRISC. Argonaute proteins can be phylogenetically separated into two clades based on sequence similarity: the Ago clade and the Piwi (P-element induced wimpy testis) clade (Carmell, 2002). Piwi proteins were first identified in a screen for factors involved in germline stem cell (GSC) maintenance in *Drosophila melanogaster* (Carmell, 2002; Lin and Spradling, 1997), a finding that was soon expanded to GSCs in other organisms (Cox et al., 1998). Several studies simultaneously reported the identification of Piwi-interacting RNAs from mouse and rat germ cells (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006). These piRNAs have emerged as an extremely complex population of small RNAs that are highly enriched in the germline tissues of the majority of metazoans analysed to date. Piwi/piRNA pathways are known to play roles in the fertility of diverse animal species, as evidenced by the fertility defects in mutants lacking Piwi (e.g. Carmell et al., 2007; Das et al., 2008; Houwing et al., 2007; Lin and Spradling, 1997). One

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well-characterised Piwi:piRNA function is the silencing of mobile elements. Such elements are autonomous pieces of DNA that replicate and insert into the genome and thus have the potential to introduce detrimental DNA damage. The regulation of mobile sequences by piRNAs canonically involves endonucleolytic cleavage ('slicing') of the target sequence after complementary base-pair recognition through the piRISC. In the germline, this process prevents accumulation of changes in the genome of the next generation and represents the most thoroughly understood aspect of piRNA biology.

Recently, several studies have started to uncover the hitherto unknown mechanisms of piRNA biogenesis. In addition, functions for piRNAs beyond transposon silencing, e.g. in regulation of mRNA, have been described. Moreover, mechanisms other than target slicing, including transcriptional regulation and mRNA deadenylation, have been described, and striking evidence for transgenerational effects of piRNAs has also been documented. In this Review, we summarise these latest advances, focussing on the mechanisms of piRNA biogenesis and modes of action of piRNAs in various organisms, including *D. melanogaster*, *C. elegans* and mice. Data from zebrafish and *Bombyx mori* are scarce but we will also occasionally draw on findings from these systems.

The birth of piRNAs

Mature piRNA sequences are surprisingly diverse between different organisms, even between closely related species. However, they share a number of characteristics other than their interaction with Piwi proteins. For example, in both *D. melanogaster* and vertebrates, piRNAs are between 26 and 30 nucleotides (nt) in length, have a 'preference' for a 5′ uracil, and posses a 3′-most sugar that is 2′-O-methylated (Kirino and Mourelatos, 2007b; Saito et al., 2007; Vagin et al., 2006). By contrast, *C. elegans* piRNAs are 21 nt long but share the 5′ and 3′ features of piRNAs in other organisms (Ruby et al., 2006). piRNA biogenesis pathways in different organisms also appear to be diverse, and are distinct from those that produce miRNAs or siRNAs, with no evidence for a double-stranded RNA precursor or a requirement for the RNAase Dicer (Das et al., 2008; Houwing et al., 2007; Vagin et al., 2006). Recent findings from *D. melanogaster*, *C. elegans* and mice have shed light on some of the players involved in regulating piRNA biogenesis and stability.

Piwi proteins and piRNA biogenesis in *D. melanogaster*

The *D. melanogaster* genome encodes three Piwi proteins, Piwi, Aubergine (Aub) and Ago3, all of which are required for male and female fertility. These Piwi proteins show distinct expression patterns: Piwi localises to nuclei in germ cells and the somatic follicle cells of the ovary; Aub is expressed in the cytoplasm of germ cells and localises partially to the nuage, an electron-dense cytoplasmically localised perinuclear structure that plays a prominent role in piRNA function; and Ago3, like Aub, is restricted to the cytoplasm of germ cells and is distinctly localised to the nuage (Brennecke et al., 2007). Even before a role for these

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factors as piRNA-binding Argonautes had been established, the study of transposable and repetitive elements in *D. melanogaster* and the phenotypes associated with the loss of these Argonautes provided valuable insights into piRNA silencing. To give one historical example, the repetitive gene locus *Stellate* is repressed in the testes of male flies by the paralogous tandem repeat Suppressor of Stellate [*Su(Ste)*] locus, and loss of *Su(Ste)* leads to crystal formation in spermatocytes (Aravin et al., 2001; Bozzetti et al., 1995). Further analysis of the *Su(Ste)* locus revealed that it expresses sense and antisense small RNAs to mediate RNAi-like silencing of the *Stellate* locus (Aravin et al., 2001). Large-scale small RNA sequencing led to the inclusion of *Su(Ste)* small RNAs into the class of repeat-associated (rasi) RNAs, which are 24-29 nt RNAs that target retrotransposons, DNA transposons, satellite and microsatellite sequences, and repetitive loci (Aravin et al., 2003). Such rasi-RNAs associate with the *D. melanogaster* Piwi proteins Piwi and Aub, and are now considered a subclass of piRNAs (Saito et al., 2006; Vagin et al., 2006).

Interestingly, different Piwi proteins bind to distinct sets of small RNAs: Piwi and Aub show a strong preference for sequences with a 5′ uridine (U) mapping antisense to transposons, whereas Ago3 piRNAs show no enrichment for 5′ U and are sense to transposons (Brennecke et al., 2007; Gunawardane et al., 2007; Saito et al., 2006; Vagin et al., 2006). Aub- and Ago3-associated piRNAs are generated by an amplification loop involving these two Piwi proteins. Aub-bound piRNAs recognise a complementary transposon transcript and induce endonucleolytic cleavage – slicing – of the target at position 10-11 of the piRNA. Such slicing generates the 5′ end of a new sense piRNA with a 10 nt 5′ overlap with the initial antisense piRNA and an adenosine residue at position 10. After 3′ end processing and modification, this new piRNA is incorporated into Ago3 and goes on to generate Aub-bound piRNAs from piRNA cluster transcripts using the same mechanism (Brennecke et al., 2007; Gunawardane et al., 2007). This cytoplasmic loop, which is also found in mice and zebrafish (Aravin et al., 2007; Houwing et al., 2007), is referred to as the 'ping-pong' cycle, and links piRNA amplification to post-transcriptional target silencing (Fig. 1A). However, this model does not account for the initial generation of primary piRNAs.

D. melanogaster piRNAs are initially derived from discrete clusters of degenerate repeat element sequences in pericentromeric and telomeric heterochromatin (Brennecke et al., 2007). These clusters can be either uni-directional, with piRNAs encoded on one strand only, or dual-stranded, with piRNAs mapping to both strands. *D. melanogaster* piRNAs are likely derived from long single-stranded precursor transcripts (Brennecke et al., 2007; Saito et al., 2007; Vagin et al., 2006). Very recently, primary piRNA biogenesis specifically from dual-stranded germline expressed clusters (Fig. 1A) has been found to require the HP1 homolog Rhino (Rhi) (Klattenhoff et al., 2009) and UAP56 (Hel25E), which colocalises with Rhi and has been linked to piRNA precursor export through the nuclear pore (Zhang et al., 2012). piRNA precursor transcripts from dual-stranded clusters are noncanonical by-products of convergent transcription of neighbouring genes. Rhi binding to H3K9me3 at these clusters mediates recruitment of the protein Deadlock and the transcription termination co-factor Cutoff, and the latter likely binds to the uncapped 5′ end of the piRNA precursor generated by 3′ end cleavage and polyadenylation of the upstream genic transcript. This prevents transcription termination, exonucleolytic degradation and splicing of the precursor (Mohn et al., 2014; Zhang et al., 2014). Designation of H3K9 trimethylated loci for Rhi binding and piRNA generation is not fully understood

but likely involves licensing by Piwi in a feed-forward loop (Mohn et al., 2014).

By contrast, transcription of uni-stranded clusters, which predominantly occurs in ovarian somatic cells (Malone et al., 2009), shows the hallmarks of canonical RNA Polymerase II (POL II) genic transcription, including defined promoter and termination sequences (Mohn et al., 2014).

Another factor that has been of outstanding interest in the context of piRNA biogenesis is the mitochondrial surface protein Zucchini (Zuc) (Pane et al., 2007). This protein has endonuclease activity for single-stranded RNA and likely processes piRNA precursors, possibly at the 5′ end (Ipsaro et al., 2012; Nishimasu et al., 2012; Saito et al., 2009; Voigt et al., 2012). Although understood in principle, the mechanism and factors underlying the maturation process from precursor to mature piRNA, including 5′ and 3′ end trimming, remain rather fuzzy. Zuc is the best candidate for 5′ processing but could alternatively be involved in 3′ end shortening and/or generation of intermediate processed RNA species. Studies in silkworm extracts, a system relatively similar to *Drosophila*, have suggested a distinct trimmer exonuclease activity for progressive 3′ end shortening of longer precursors but no candidate factor has yet been identified (Kawaoka et al., 2011).

Several large-scale RNAi screens using qPCR-based analysis of transposon expression or *LacZ* reporters for either somatic or germ cell piRNA pathway components have also identified factors involved in piRNA biogenesis (Czech et al., 2013; Handler et al., 2013; Muerdter et al., 2013). One such factor is the uncharacterised protein CG2183 (Gasz), which localises with Zuc to mitochondria and likely acts as an adapter that recruits a complex of Armitage (a non-DEAD-box helicase) and Piwi to mitochondria for piRNA loading and maturation (Czech et al., 2013; Handler et al., 2013). CG2183 is the *D. melanogaster* ortholog of murine GASZ (ASZ1), a protein involved in piRNA-mediated silencing of retrotransposons (Ma et al., 2009). The piRNA methyltransferase Pimet (Hen1), the homolog of *Arabidopsis* methyltransferase HEN1, is required for 2′-O-methylation of maturing piRNAs (Saito et al., 2007), a conserved step in piRNA maturation. A description of all known *D. melanogaster* piRNA biogenesis factors would be beyond the scope of this work and has been reviewed elsewhere (Guzzardo et al., 2013). One prominent class of proteins worth mentioning, however, is the family of tudor-domain proteins (TDRDs), which work as scaffolds for proteins carrying symmetrically methylated arginine residues. TDRDs have, among other functions (reviewed by Pek et al., 2012), been linked to primary piRNA biogenesis (Handler et al., 2011).

PIWI proteins and piRNA biogenesis in *M. musculus*

The mouse genome encodes three Piwi proteins, MIWI (PIWIL1), MILI (PWIL2) and MIWI2 (PWIL4), all of which are individually required for male but not female fertility (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). They are each expressed at different stages during development, with MILI expression starting at embryonic day (E) 12.5, after primordial germ cells (PGCs) have migrated into the developing gonad, and persisting into adulthood. By contrast, MIWI2 expression occurs later and is limited from 15 dpc (days post coitum) to 3 dpp (days post partum), a time window that correlates with cell cycle arrest and *de novo* methylation in PGCs. MIWI is expressed in adult testes from 14 dpp, coinciding with the onset of the pachytene stage of meiosis (Aravin et al., 2008). The phenotypes found in *Mili* and *Miwi2* knockout animals manifest early during spermatocyte meiosis, whereas in *Miwi* mutants spermiogenesis is impaired at the later round spermatid stage
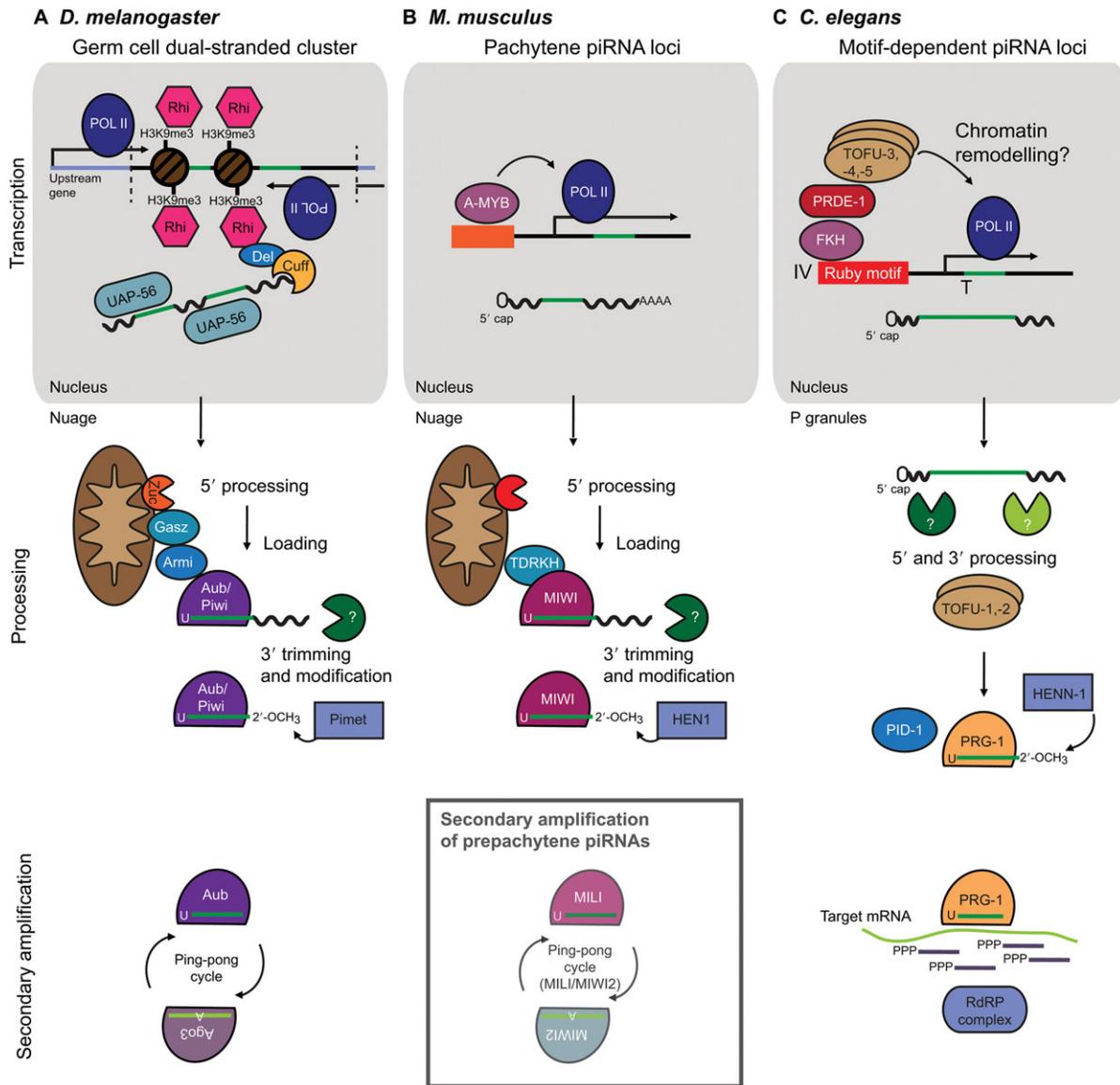
Fig. 1. Mechanisms of piRNA biogenesis in different organisms. (A-C) Models of piRNA generation from dual-stranded clusters in *D. melanogaster* (A), from pachytene piRNA loci in *Mus musculus* (B) and from Ruby motif-containing loci in *C. elegans* (C). (A) Convergent transcription from neighbouring genic loci generates piRNA precursors from dual-stranded clusters upon binding of the heterochromatin protein Rhino (Rhi) to H3K9me3 on cluster loci. Rhi in turn associates with Deadlock (Del) and Cutoff (Cuff), the latter of which is thought to protect the 5′ end of the non-canonical precursor transcript from degradation. Nuclear export of the piRNA, mediated by UAP-56, is followed by 5′ end processing, likely mediated by mitochondria-associated nuclease Zucchini (Zuc). Additional factors [e.g. CG2183 (Gasz) and Armitage (Armi)] lead to Piwi protein recruitment, piRNA loading and 3′ end processing, which likely involves an unknown trimmer activity as well as the action of methyltransferase Pimet. Extensive secondary piRNA amplification occurs via the ping-pong cycle, which takes place in *Drosophila* germ cells. (B) The transcription factor A-MYB (MYBL1) binds to a canonical promoter motif and initiates piRNA precursor transcription by POL II while simultaneously inducing expression of piRNA pathway genes (e.g. *Miwi* and *Mitopld*). The precursor transcripts are 5′ capped and poly-A-tailed and, after export from the nucleus, processed by the murine homolog of Zuc, MITOPLD. Loading onto MIWI is likely followed by 3′ end trimming of the precursor and 2′-O-methylation by murine HEN1. For conceptual comparison of secondary amplification mechanisms in different organisms, the MILI-MIWI2 ping-pong cycle occurring only for prepachytene piRNAs is included here (inset). This process does not take place as part of the biogenesis of pachytene MIWI-bound piRNAs shown here. (C) The conserved Ruby motif is bound by Forkhead proteins (FKH) and possibly additional factors and transcription of 5′ capped 28 or 29 nt precursors is initiated. Transcription and/or stability of these precursors depend on PRDE-1, TOFU-3, TOFU-4 and TOFU-5. After 5′- and 3′ end processing of the precursor, a process that may be mediated by TOFU-1 and TOFU-2, 2′-O-methylation of the 3′ end of the piRNA by HENN-1 takes place. PID-1 is another novel factor involved in piRNA biogenesis or stabilisation, possibly acting at the same level as the *C. elegans* Piwi protein PRG-1. PRG-1:piRNA:target RNA interaction leads to the generation of secondary 22G-RNAs carrying a 5′ triphosphate (indicated as PPP) by a multi-protein machinery containing RNA-dependent RNA polymerases (RdRP). These small RNAs are incorporated into a secondary Argonaute and mediate target silencing. Question marks indicate unknown factors or functions; green lines represent piRNA sequences; blue lines represent upstream sequences. Although the role of *D. melanogaster* UAP-56 in the targeting of piRNA precursors for nuclear export has been described, analogous mechanisms in mice and *C. elegans* have not yet been discovered; therefore, the sequence of events showing export of a long precursor from the nucleus to the cytoplasm in these organisms is speculative. Biogenesis of uni-stranded clusters in *D. melanogaster*, which occurs concomitantly with dual-strand cluster expression in germ cells and is the only mode of piRNA generation in somatic follicle cells, may be similar to canonical POL II transcription of protein-coding genes. It is currently less well studied and not depicted here. The same is the case for primary biogenesis of prepachytene piRNAs, which are expressed in the foetal germline in *M. musculus*, and for Ruby motif-independent piRNAs, which make up a small proportion of the overall piRNA population in *C. elegans* adults. Ago3, Argonaute3; Aub, Aubergine; RdRP, RNA-dependent RNA polymerase; TDRKH, a tudor domain protein.

(Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). In addition to and independently of their distinct expression patterns, murine PIWI proteins are associated with distinguishable subsets of piRNAs: MILI-bound piRNAs are 26 or 27 nt; MIWI2-bound piRNAs are slightly longer at 28 nt; and MIWI-bound piRNAs peak at 30 nt (Aravin et al., 2008, 2006; Girard et al., 2006).

Different populations of piRNAs in mice have also been distinguished based on their expression pattern during development: pre-pachytene piRNAs are predominantly present in the germ cells of foetal and newborn mice, are enriched for transposon and gene-derived sequences, and bind to MILI and MIWI2; pachytene piRNAs originate from distinct intergenic loci, are depleted of repeat sequences, and associate with MILI and MIWI (Aravin et al., 2008; Li et al., 2013). For this latter class of piRNAs, a transcriptional master regulator – A-MYB (MYBL1) – has been identified (Fig. 1B): A-MYB induces POL II-mediated transcription of both long pachytene piRNA precursors (defined here as sequences longer than 100 nt but often substantially longer) and pathway proteins, including MIWI and MITOPLD in a concerted manner (Li et al., 2013). Interestingly, MITOPLD, also called PLD6, is a phospholipase and the mouse homolog of the *D. melanogaster* piRNA biogenesis factor Zuc. Initially, MITOPLD was thought to act on the piRNA pathway by modulating lipid signalling at the outer mitochondrial membrane (Huang et al., 2011; Watanabe et al., 2011a). However, recent studies on MITOPLD and on *D. melanogaster* Zuc have revealed nuclease rather than phospholipase activity (Ipsaro et al., 2012). A recent study by Shiromoto and colleagues has identified the outer mitochondrial membrane protein GPAT2, which is a glycerol-3-phosphate-acetyltransferase, as a MILI interaction partner that is required for primary piRNA biogenesis independently of its catalytic activity (Shiromoto et al., 2013). The *in vivo* function of this protein awaits further investigation; however, it is interesting to note that the link between the piRNA pathway and mitochondria is conserved from insects to mammals (Fig. 1A,B). Moreover, mouse knockout models of murine homologs of *D. melanogaster* CG2183 (GASZ) and Armitage (MOV10) display severely reduced levels of piRNAs, hinting at similar mechanistic function for these factors in both organisms (Czech et al., 2013; Handler et al., 2013; Ma et al., 2009; Zheng et al., 2010).

The analysis of mouse MIWI- and MILI-associated sequence tags (Vourekas et al., 2012) has shown that 3′ end extended sequences associate with Piwi proteins, indicating that 5′ end processing and incorporation of the 5′ U into the MID domain of the Piwi protein occur first. This is likely followed by 3′ end trimming by an unidentified exonuclease, by 3′ end 2′-O-methylation of the piRNA by the mouse homolog of HEN1 (HENMT1) and, finally, by binding of the 3′ end by the PAZ domain of the Piwi protein (Kirino and Mourelatos, 2007a,b). Conceptually, this model, in which an exonuclease processes piRNA precursors from the 3′ end, may also hold true for *Drosophila* and is further supported by *in vitro* studies of silkworm germ cell extracts, which identified trimmer activity (Kawaoka et al., 2011). In the context described here, the extent of 3′ end trimming and consequently piRNA length is determined by the Piwi protein, possibly explaining the different size profiles of piRNAs associated with distinct Piwis. Interestingly, in mice, another tudor domain protein, TDRKH, which interacts with di-methylated MIWI and MIWI2 in mitochondria, has been implicated in the final 3′ precursor maturation step (Saxe et al., 2013).

Piwi proteins and piRNA biogenesis in *C. elegans*

Caenorhabditis elegans piRNAs are also referred to as 21U-RNAs due to their unusual length of 21 nt and their bias for a 5′ U. They are bound to PRG-1, the single functional *C. elegans* Piwi homolog (Batista et al., 2008; Das et al., 2008; Wang and Reinke, 2008). PRG-1 is essential for the presence of 21U-RNAs, although a direct role for this Piwi protein in piRNA biogenesis rather than stability appears unlikely as vey low levels of mature piRNAs are still detectable in *prg-1* mutant animals (Das et al., 2008). Another Piwi gene encoded in the *C. elegans* genome, *prg-2*, likely has little or no function in the piRNA pathway (Batista et al., 2008; Das et al., 2008).

The majority of the >16,000 piRNA genes of *C. elegans* are found in two clusters on chromosome IV, with each piRNA located downstream of a distinctive bipartite sequence motif (Ruby et al., 2006). For brevity, we refer to this motif as the Ruby motif after the first author of the paper describing this sequence upstream of 21U-RNAs. Recent experimental data have shown that this sequence acts as an autonomous promoter for individual piRNA precursors (Fig. 1C) (Billi et al., 2013; Cecere et al., 2012). Of note, a subset of small RNAs bound to PRG-1 are generated from sites outside the canonical clusters and do not depend on the Ruby motif (Gu et al., 2012b; Weick et al., 2014). The 21U-RNA precursor transcripts carry a 5′ 7-methylguanylate cap and are likely generated by POL II, with transcription starting exactly 2 nt upstream of the 5′ U of the mature piRNA. There has been some debate regarding the overall length of the precursors: Cecere et al. have detected a >60 nt capped transcript using 5′ RACE, whereas Gu et al. detected shorter 26 nt putative precursor species by small RNA sequencing (Cecere et al., 2012; Gu et al., 2012b). We and others have recently refined this finding showing that it is indeed short species of 28 or 29 nt that are made from most piRNA loci (Goh et al., 2014; Weick et al., 2014).

The transcription of piRNAs from the Ruby motif is at least in part regulated by redundantly acting Forkhead proteins, including UNC-130, FKH-3, FKH-4 and FKH-5. Upon depletion of these germline-expressed proteins by RNAi knockdown or gene knockout, several mature piRNAs displayed decreased levels when assessed by qPCR (Cecere et al., 2012). Moreover, *in vitro* interaction between the Ruby motif and FKH-3, FKH-5 and UNC-130 has been shown, and further experiments have confirmed this for UNC-130 *in vivo* (Cecere et al., 2012). As the Forkhead family is widely involved in transcriptional regulation, the question remains as to how specificity of the transcription machinery for the Ruby motif and the generation of short piRNA precursors is achieved. Several recent reports have begun to shed light on this. Our laboratory has identified piRNA defective 1 (PRDE-1) as a novel factor that is essential for the generation of Ruby-motif dependent piRNA precursors and accumulates in pachytene germ cell nuclei on chromosome IV. PRDE-1 is either involved in generating these precursors by direct or indirect interaction with the motif or may stabilise the precursors once they have been transcribed from the motif (Weick et al., 2014). Furthermore, using an RNAi based genome-wide screen, the Hannon lab has identified several other factors required for *C. elegans* piRNA biogenesis, which they collectively refer to as TOFUs for 'twenty-one U fouled up' (Goh et al., 2014). Although further work will be required to investigate the localisation, interactions and mechanisms of these proteins, analysis of the precursor and mature piRNA populations following perturbation of these TOFUs has provided valuable insights into the hierarchy of the biogenesis mechanism. In brief, *tofu-3/ulp-5*, *tofu-4* and *tofu-5* are required for precursor production and, based on their predicted domain structures, might be involved in chromatin remodelling. By contrast, *tofu-1* and *tofu-2* mutants lack mature 21U-RNAs and display elevated levels of precursor RNAs, indicating a role for these factors in precursor processing. Finally,

a third report has identified *pid-1*, a cytoplasmic factor with unknown function, as an essential factor for piRNA biogenesis in *C. elegans* (de Albuquerque et al., 2014). Animals lacking *pid-1* express piRNA precursors but display strongly reduced numbers of mature piRNAs. However, 2′-O 3′ end modification by the *C. elegans* HEN1 ortholog HENN-1 (Kamminga et al., 2012; Montgomery et al., 2012), which is assumed to be involved in one of the last steps of piRNA maturation, is not affected in the remaining mature piRNAs. Based on the reduction in mature, rather than precursor, piRNAs and the cytoplasmic localisation of PID-1, this protein may function by interacting with PRG-1 directly.

Taken together, these data provide an exciting starting point for further understanding the unique mechanisms of piRNA biogenesis and stability in *C. elegans*. Strikingly, and despite the clear conservation of the Piwi protein itself, no piRNA biogenesis factor that is conserved between *C. elegans* and *D. melanogaster* or mice has been identified to date. Furthermore, the ping-pong mechanism, which serves to amplify piRNAs in flies and mice, does not occur in nematodes (Das et al., 2008). Interestingly, worms employ a distinct analogous signal amplification mechanism that leads to generation of secondary downstream siRNAs upon piRNA: target RNA interaction (on which more below). Despite these apparent differences in the nematode pathway, other protein factors found in *Drosophila* and mice have also been implied in piRNA silencing in *C. elegans*: tudor-domain proteins have a demonstrated role in endogenous small RNA pathways (Thivierge et al., 2011) and EKL-1 is a TDRD involved in piRNA-dependent siRNA generation (Gu et al., 2009). The *C. elegans* genome encodes several additional uncharacterised TDRDs and study of their involvement in the piRNA pathway should be an interesting avenue for future research.

Mechanisms of piRNA-mediated transcriptional silencing

Mobile elements are the most prominent piRNA targets, and the cytoplasmic ping-pong cycle, in which a transposon target is recognised by a piRNA and sliced by the Piwi protein via its catalytic domain to give rise to a new piRNA with opposite orientation, is a particularly well-understood post-transcriptional mechanism for transposon repression. Many members of the Piwi protein family have a conserved catalytic domain and are therefore capable of target 'slicing'. The identification of the ping-pong cycle in *D. melanogaster* and mice as an efficient means for both transposon transcript degradation and small RNA amplification clearly showed the requirement for a cytoplasmic component in piRNA silencing. Nevertheless, nuclear localisation of *D. melanogaster* Piwi and murine MIWI2 provided strong evidence for additional modes of silencing (Aravin et al., 2008; Brennecke et al., 2007). Below, we discuss some of the most recent reports indicating a role for transcriptional silencing as a mechanism for piRNA-mediated silencing of transposons and exogenous transgenes. Additional mechanisms of piRNA action, namely target mRNA deadenylation, have also been reported, however, as they are implied in regulation of protein-coding gene expression rather than transposon silencing, we discuss these findings later when describing new evidence for non-transposon targets of piRNAs.

Evidence for piRNA-mediated transcriptional silencing in
D. melanogaster
Several studies have explored the role of *D. melanogaster* Piwi in transcriptional gene silencing (TGS). Interestingly, studies have shown that Piwi nuclear localisation but not its slicer activity is required for the silencing of transposable elements (Klenov et al.,

2011; Saito et al., 2010). In addition, loss of Piwi leads to loss of histone H3 lysine 9 trimethylation (H3K9me3) and an increase in POL II occupancy at transposable elements (Le Thomas et al., 2013; Sienski et al., 2012); and recruitment of the heterochromatin protein HP1 to a piRNA reporter subjected to TGS has also been demonstrated (Le Thomas et al., 2013). Together, these findings suggest a model for Piwi-mediated TGS in which Piwi translocates to the nucleus to potentially interact with nascent transcript or DNA at the target locus, which in turn leads to heterochromatin formation and transcriptional repression (Fig. 2A). piRISC-induced TGS also requires the zinc-finger protein GTSF-1/Asterix, which likely directly interacts with Piwi and is required for establishment of H3K9 methylation (Donertas et al., 2013; Handler et al., 2013; Muerdter et al., 2013). Furthermore, histone methylation may not be the final silencing mark; the high mobility group protein Maelstrom, like Piwi, is required for POL II inhibition; however, changes in H3K9me3 methylation following *mael* knockdown are modest compared with the effects seen upon *piwi* knockdown, indicating that Mael acts downstream of Piwi and histone methylation (Sienski et al., 2012). The exact role of Mael remains to be determined, but either DNA binding via its HMG box domain or RNA binding via the RNAse H fold in its Mael domain may be envisaged (Zhang et al., 2008).
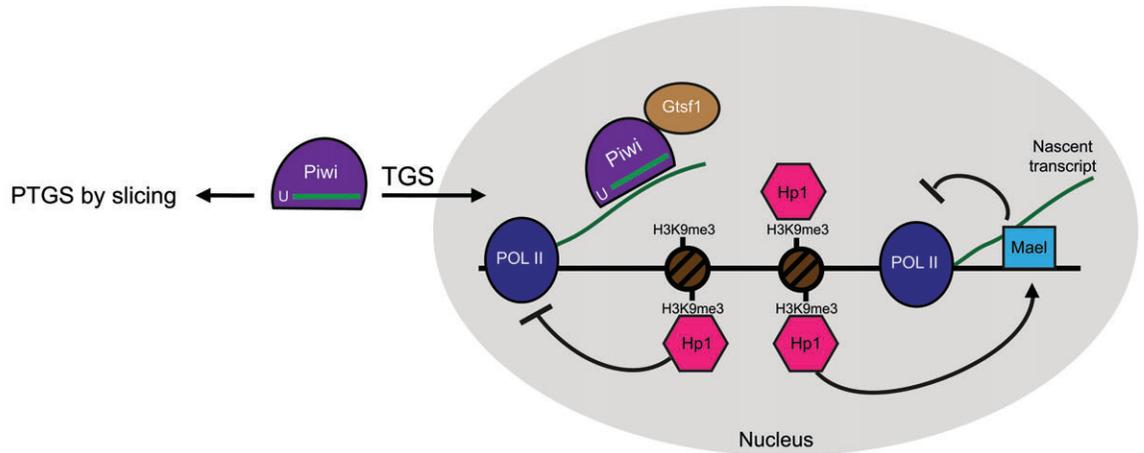
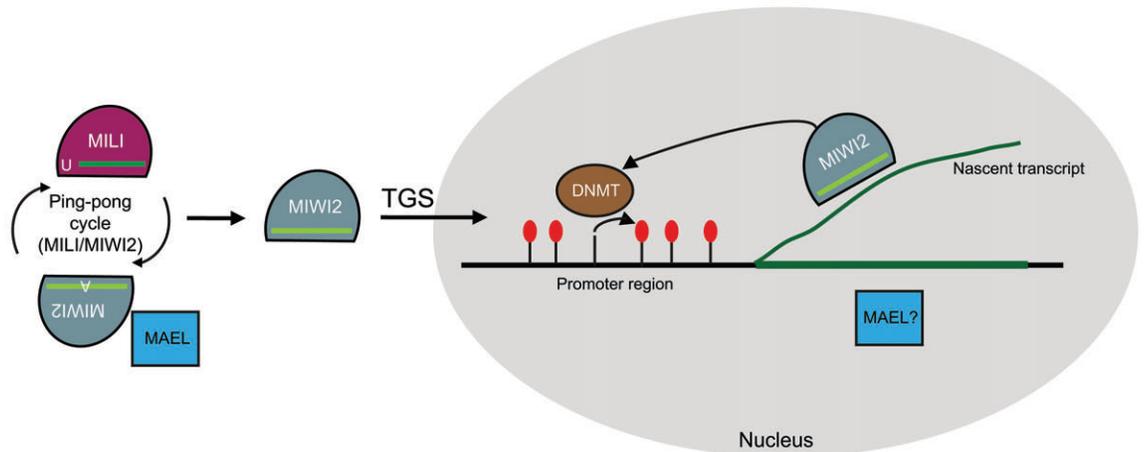### Evidence for piRNA-mediated transcriptional silencing in
M. musculus
Evidence for transcriptional, as well as post-transcriptional, piRNA-mediated silencing is not limited to *D. melanogaster*. In mice, MILI and MIWI2 act together to promote the establishment of retrotransposon silencing by CpG DNA methylation in the male mouse foetal germline (Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008). This involves binding of transposon-derived primary piRNAs to MILI and the generation of secondary piRNAs by ping-pong amplification, either by MILI-MILI or by MILI-MIWI2 interactions in the nuage of germ cells (Aravin et al., 2008; De Fazio et al., 2011). In line with this, MIWI2 expression in the male germ line is restricted to the narrow time window in which cell cycle arrest and *de novo* methylation occur in PGCs. As MIWI2 localises to the nucleus as well as the cytoplasm, it has been suggested to shuttle to the nucleus to mediate DNA methylation-dependent TGS once it has been loaded with secondary piRNAs (Fig. 2B). Interestingly, *Mael*, which acts downstream of Piwi in *D. melanogaster*, is highly conserved in mice and is found in cytoplasmic structures with MIWI2. Furthermore, *Mael* mutant animals display some moderate defects in DNA methylation in foetal gonocytes and during adult meiosis (Aravin et al., 2009; Soper et al., 2008). However, whether MAEL plays a role in downstream transcriptional silencing similar to that in flies remains to be investigated.

MIWI2 is de-localised in *Mili* mutant mice, showing that MILI acts epistatic to MIWI2 (Aravin et al., 2008). However, MIWI2 slicer activity is not required for the silencing of LINE-1 (L1) elements and, in fact, MIWI2 catalysis-deficient mutants are fertile and repress transposable elements to wild-type levels (De Fazio et al., 2011). By contrast, transposable element repression by *de novo* DNA methylation of L1 during foetal development requires MILI catalysis, as shown in a MILI slicer-dead mutant (De Fazio et al., 2011; Di Giacomo et al., 2013). However, the requirement for MILI-mediated endonucleolytic cleavage (and ping-pong amplification) is restricted to highly expressed transposons such as L1 but does not apply to the IAP element, which makes up a much smaller proportion of the mouse genome (De Fazio et al., 2011).
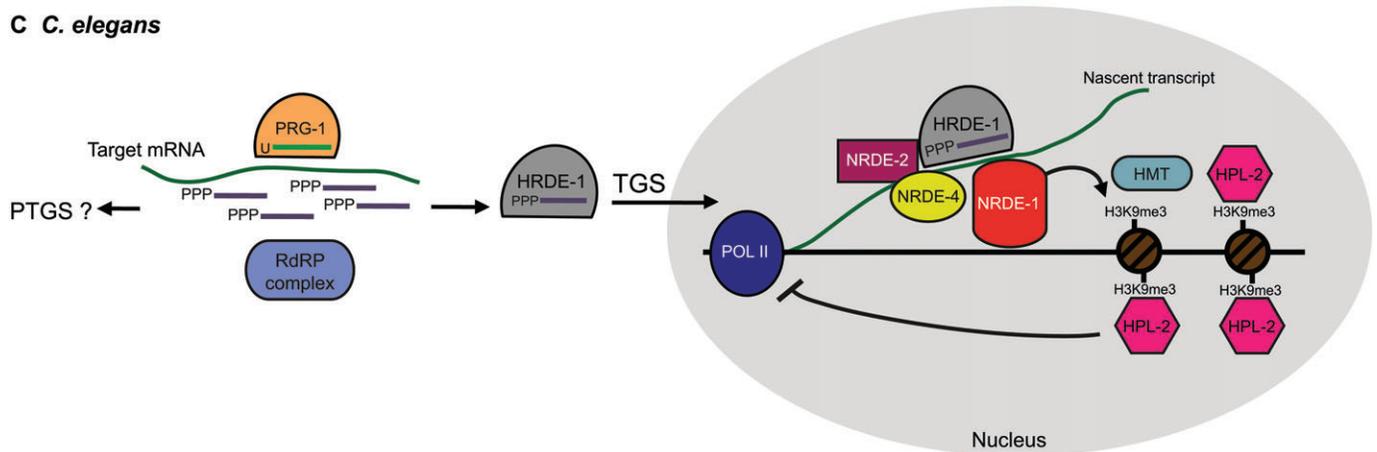
Fig. 2. Mechanisms of piRNA-mediated transcriptional silencing. (A) In *D. melanogaster*, Piwi localises to the nucleus and initiates repressive histone H3K9 trimethylation and RNA polymerase II stalling. Whether Piwi interacts with the nascent transcript or directly with DNA is not understood. The zinc-finger protein Gtsf1 likely directly interacts with Piwi, whereas the heterochromatin protein Hp1 binds to H3K9me3. Mael acts downstream of H3K9me3 methylation and is required for POL II repression; however, its mechanism of action also remains to be determined. In parallel to transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS; i.e. slicing) plays a well-defined role in *D. melanogaster* piRNA-mediated transposon silencing. (B) In *M. musculus*, MIWI2 engages in the ping-pong cycle with MILI and translocates to the nucleus where it initiates CpG methylation of promoter elements upstream of transposon loci by DNA methyltransferase (DNMT) action. The murine MAEL homolog is found in the cytoplasm at MIWI2 sites; a role for this protein in the nucleus analogous to that described in *D. melanogaster* remains to be determined. Interaction of MIWI2 with the nascent transcript is speculative. (C) Secondary siRNAs are generated upon PRG-1:piRNA:target RNA interaction by RNA-dependent RNA polymerase (RdRP). These small RNAs are incorporated into the germline secondary Agonaute HRDE-1 which translocates to the nucleus to initiate H3K9me3 methylation and POL II stalling, likely by interacting with pre-mRNA and nuclear RNAi (NRDE) factors. For clarity, this model depicts establishment of repressive histone methylation marks by histone methyltransferases (HMT) as preceding POL II repression; the actual order of events remains to be determined experimentally and the reverse may also be the case. In *C. elegans,* target slicing is not essential for piRNA function; however, other mechanisms of PTGS have not been experimentally investigated to date. HPL, a H3K9me3-binding protein.

During adult germ cell meiosis, MILI is required for only post-transcriptional silencing of L1 elements, with DNA methylation occurring in a piRNA-independent manner, indicating that TGS and post-transcriptional gene silencing (PTGS) go hand in hand in the mouse germline (Di Giacomo et al., 2013). Further highlighting the role of PTGS is the fact that MIWI, the third murine Piwi protein, which is expressed in adult meiotic sperm cells, mediates L1 repression via transposon slicing (Reuter et al., 2011). Unlike MILI- and MIWI2-bound piRNAs, MIWI piRNAs show only a weak ping-pong signature, and secondary amplification of piRNAs likely does not play a prominent role in adult testes. Unlike the prepachytene piRNAs described here, the majority of pachytene piRNAs do not map to repeat elements and do not engage in transposon silencing by target slicing or TGS. Instead, these piRNAs have very recently been implicated in deadenylation-mediated mRNA degradation. We will discuss this distinct mechanism of PTGS below when presenting evidence for non-transposon targets of the piRNA pathway.

Evidence for piRNA-mediated transcriptional silencing in *C. elegans*
The crucial role for TGS as a downstream consequence of piRNA action is further supported by data from *C. elegans*. Like *Drosophila melanogaster*, nematodes do not exhibit canonical CpG methylation. However, silencing by histone modifications and transcriptional repression via POL II stalling has a well-documented role in exogenous and endogenous small RNA pathways in the somatic tissues of *C. elegans* (Burkhart et al., 2011; Grishok, 2005; Guang et al., 2010, 2008). Below, we first outline the somatic nuclear RNAi pathway in *C. elegans*, then relate back to the most recent findings in piRNA-mediated TGS, which likely employs a very similar mechanism.

In *Caenorhabditis elegans*, the transmission of different small RNA pathways occurs as a two-step mechanism whereby target recognition is followed by the generation of secondary siRNAs by RNA-dependent RNA polymerases (RdRPs). These secondary siRNAs, also known as 22G-RNAs, are then incorporated into a downstream Argonaute protein that mediates target silencing. In somatic nuclear RNAi, this downstream Argonaute, nuclear RNAi-deficient 3 (NRDE-3) shuttles into the nucleus where it associates with pre-mRNA and recruits the uncharacterised NRDE-2 protein (Guang et al., 2008, 2010). Both NRDE-3 and NRDE-2 do not bind chromatin directly; however, they are required for recruitment of another protein, NRDE-1, to chromatin and for subsequent repressive H3K9me3 methylation at the target site (Burkhart et al., 2011). The exact mechanism of transcriptional silencing remains unknown; however, nuclear RNAi was shown to mediate inhibition of POL II during the elongation phase of transcription (Guang et al., 2010).

The *C. elegans* piRNA pathway, which functions exclusively in the germline, also mediates silencing via RdRP-dependent generation of secondary 22G-RNAs (Bagijn et al., 2012; Das et al., 2008; Lee et al., 2012). Interestingly, this provides a target-based amplification loop that is similar to some extent to that occurring as part of the ping-pong cycle. We and others have recently found that the piRNA pathway mediates gene silencing at the pre-mRNA level (Bagijn et al., 2012) and that silencing depends on a number of chromatin factors, including the *C. elegans* homolog of the H3K9me3-binding protein HP1, HPL-2, and several histone methyltransferases (Ashe et al., 2012; Shirayama et al., 2012). The establishment of this nuclear silencing downstream of piRNAs occurs by a mechanism that is very similar to the relatively well-characterised nuclear RNAi pathway acting in somatic tissues: a

germline-specific nuclear Argonaute known as HRDE-1 (heritable RNAi-deficient 1) binds secondary 22G-RNAs and likely functions in a manner that is analogous to the somatic NRDE-3 (Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012), shuttling into the nucleus and initiating H3K9me3 methylation and POL II stalling (Fig. 2C). piRNA-mediated TGS also depends on NRDE-1, NRDE-2 and NRDE-4, indicating that these are general rather than soma-restricted nuclear small RNA pathway factors.

Despite the identification of some of the factors involved in piRNA-mediated TGS in animals, much remains to be learned about the role of piRNAs in translating a small RNA signal into gene repression, in particular because these mechanisms are likely fundamentally different from the more extensively studied TGS pathways of *Schizosaccharomyces pombe* and plants (reviewed by Castel and Martienssen, 2013).

piRNA functions beyond transposon silencing
Although the role of piRNAs in silencing repeat elements is well established, evidence from various organisms has identified scores of piRNAs that do not readily match to transposons or repetitive pseudogenes. Accordingly, recent reports have found the exciting potential for a targeting repertoire that extends far beyond transposons and, excitingly, also employs a distinct mode of piRNA-mediated repression.

Repression of protein-coding targets by the Piwi/piRNA pathway in *D. melanogaster*
Although the majority of piRNAs in *D. melanogaster* can be mapped to degenerate repeat elements (Brennecke et al., 2007), two publications in 2009 reported expression of sense piRNAs from the 3′ UTRs of genes in *D. melanogaster* somatic follicle cell lines (Robine et al., 2009; Saito et al., 2009). The conclusions regarding the modi operandi for these non-TE piRNAs were contradictory. Saito et al. postulated regulation *in trans* of the protein-coding transcript *Fas3* by piRNAs generated from the 3′ UTR of the *traffic jam* transcript, whereas Robine et al. found that Traffic Jam protein levels were elevated in *piwi* mutants, indicating a cis-regulatory mechanism for piRNA action. Follow up studies on these mRNA-derived piRNAs should clarify the mechanism by which they mediate target silencing. Interestingly, a study examining *Nanos* (*nos*) mRNA deadenylation in the *D. melanogaster* embryo showed that transposon-derived piRNAs can target protein-coding mRNAs *in trans* with incomplete complementarity (Rouget et al., 2010). Here, the CCR4-NOT deadenylation complex, which is responsible for degradation of maternal *nos* mRNA in the majority of the embryo, regulates embryonic patterning and interacts directly with the Piwi proteins Aub and Ago3. Moreover, *nos* deadenylation depends on piRNA target sites in the 3′ UTR of the *nos* transcript, providing striking evidence for a silencing mechanism that is distinct from 'slicing' and TGS. These findings in flies open up many routes for further investigation, both with regards to the potential of piRNAs to silence genes using several different mechanisms and also in terms of increasing the target repertoire of piRNAs tremendously, as perfect sequence complementarity between the piRNA and its target are not required for efficient repression by deadenylation.

Repression of protein-coding targets by the Piwi/piRNA pathway in *M. musculus*
In the murine system, the role of pachytene piRNAs, which make up the abundance of piRNAs in adult male germ cells but do not map to transposable elements, has long remained mysterious. Based on

co-fractionation assays and HITS-CLIP experiments, which use high-throughput sequencing of RNAs after crosslinking to their protein interaction partners, Vourekas et al. have recently proposed a model whereby pachytene piRNAs, rather than serving as sequence guides for repression, are generated as part of a clearance process for long non-coding RNAs in spermiogenesis (Vourekas et al., 2012). Here, the process of piRNA biogenesis becomes a degradation mechanism in itself. This study also identified MIWI complexes containing spermiogenic mRNAs but no piRNAs at the late round spermatid stage. As these mRNAs are decreased in *Miwi* mutant animals, the Piwi protein may play a stabilising rather than repressive role. These striking observations seem to contradict most of what we know about the function of Argonaute proteins in general and the silencing action of piRNAs in particular. Other studies have come to different conclusions, some of which contradict the findings of Vourekas et al.: regarding a role in mRNA stabilisation, Reuter et al. previously showed that mRNAs, which are reduced in a *Miwi* knockout background at the round spermatid stage in a piRNA-independent manner, are also equally reduced in a *Miwi* slicer-dead background (Reuter et al., 2011). As MIWI lacking its catalytic domain is unable to stabilise these mRNAs, their reduction is likely a consequence of the developmental block observed in *Miwi*-null as well as in *Miwi* slicer-dead mutant animals, rather than a result of the loss of a direct stabilising interaction with MIWI. A recent study analysing the role of pachytene piRNAs in elongating spermatids, a later stage of spermatogenesis, found a striking role for pachytene piRNAs in mediating the concerted degradation of the bulk of cellular mRNAs (Gou et al., 2014). This process involves target recognition by imperfect base-pairing and, rather than slicing, employs mRNA deadenylation via interactions between MIWI and CAF1, a major catalytic subunit of the CCR-4-CAF-1-NOT deadenylase complex, to promote target elimination in a process similar to that described in flies (Gou et al., 2014; Rouget et al., 2010). The apparent discrepancies regarding the postulated roles of MIWI and pachytene piRNAs between these different studies are in part explained by the different time points examined in the respective papers: MIWI may contribute to stabilisation of a subset of mRNAs in a piRNA-independent manner, either directly or indirectly, during the round spermatid stage and then contribute to mRNA deadenylation in a piRNA-dependent manner at the later elongating spermatid stage.

Repression of protein-coding targets by the Piwi/piRNA pathway in *C. elegans*

Unlike piRNAs in other organisms, *C. elegans* 21U-RNAs do not readily map to transposable element remnants (Batista et al., 2008; Das et al., 2008; Wang and Reinke, 2008). This finding was particularly puzzling initially as the *C. elegans* Piwi *prg-1* is clearly required for transposon silencing (Das et al., 2008). However, further analysis revealed that nematode piRNAs, rather than requiring perfect complementarity, are capable of silencing *in trans* by imperfect base-pairing, allowing for further definition of the piRNA target spectrum (Bagijn et al., 2012; Lee et al., 2012). Besides transposons, a number of protein-coding genes are silenced by 21U-RNAs, likely by the same TGS mechanism described above. The biological and phenotypic relevance of individual target mRNA:piRNA silencing relationships is difficult to analyse. However, we have recently found that distinct classes of piRNAs employ different biogenesis mechanisms, being either dependent on PRDE-1 and the Ruby motif or being generated by an independent mechanism (Weick et al., 2014). The identification of these

differing requirements allowed for differential analysis of these subsets and their effects on expression of protein-coding targets. Interestingly, unlike motif-dependent piRNAs, motif-independent piRNAs show enrichment of immune-response genes among their protein-coding targets, suggesting a biologically distinct function for this class of 21U-RNAs.

piRNAs as mediators of transgenerational effects
Evidence for piRNA-mediated transgenerational inheritance in *C. elegans*

Recent studies have shown that, strikingly, the *C. elegans* nuclear Argonaute HRDE-1, which binds the 22G-RNAs generated downstream of piRNAs, then shuttles into the nucleus and initiates H3K9me3 methylation and POL II stalling, can mediate transgenerational epigenetic silencing (Fig. 3A). This silencing remains stable even in the absence of the original PRG-1:piRNA trigger (Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Once silenced, an epi-allele generated via this mechanism can act in a dominant manner, turning off other previously expressed alleles (Shirayama et al., 2012). A role for heterochromatin formation at a heritably silenced piRNA reporter locus has been confirmed (Luteijn et al., 2012), and stably silenced transgenes for which induction of silencing likely depends on the Piwi protein PRG-1 also display repressive chromatin marks (Shirayama et al., 2012).

Although silencing can become independent of the Piwi/piRNA trigger, data from a piRNA-independent transgenic sensor for heritable silencing show that 22G-RNAs targeting this sensor persist into at least the F4 generation (Ashe et al., 2012). Moreover, time course studies of heritable silencing after dsRNA-induced RNA interference, a process that depends on the same downstream factors involved in long-term piRNA-mediated silencing, have shown that, in this context, small RNAs precede the onset of H3K9me3 chromatin modification (Gu et al., 2012a). How exactly the silencing signal is transmitted from one generation to another in remains to be determined. Direct transmission of parental piRNAs to the embryo followed by primed amplification is an elegant mechanism for re-establishing silencing in each generation. Such a mechanism can indeed be found in *D. melanogaster* (discussed below); however, it cannot fully explain the transgenerational effects observed in *C. elegans*. Here, 21U-RNAs and PRG-1 may be transmitted to the embryo, yet, as silencing can become independent of the piRNA trigger, other mechanisms must be in place to propagate silencing. One might envisage that secondary 22G-RNAs could be passed on through generations to initiate heterochromatin formation. This would require amplification of the 22G-RNA signal in each generation. Alternatively, chromatin marks could be the inherited mark priming 22G-RNA production anew every generation. Although the data described above argue that this is not the case for repressive H3K9 methylation (Gu et al., 2012a), profiling of other chromatin marks in inheritance phenomena has not been carried out so far.

Evidence for piRNA-mediated transgenerational inheritance in *D. melanogaster*

In *Drosophila*, intercrosses between strains in which the paternal genome contains active transposons not expressed in the mother can lead to infertile daughters; this phenomenon is called hybrid dysgenesis. In this context, maternally deposited piRNAs initiate piRNA production by providing the antisense piRNA component of the ping-pong loop, which mounts a defence response in concert with the sense piRNA component provided by degenerate copies of
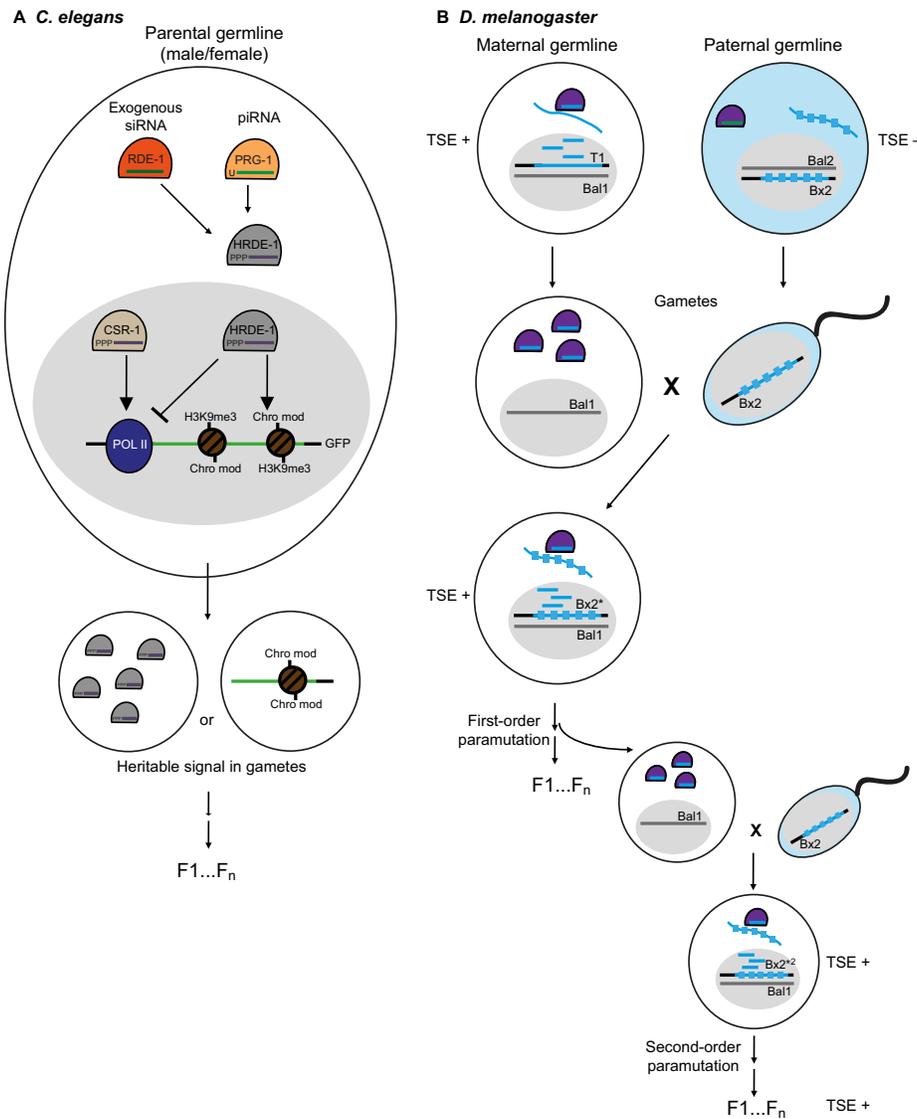
Fig. 3. piRNA-mediated transgenerational silencing in *C. elegans* and *D. melanogaster*. (A) Stable transgenerational silencing of transgenes can be induced by piRNAs and exogenous siRNAs (RNAi) in the *C. elegans* germline. Small RNA:target RNA interaction leads to generation of secondary siRNAs (22G-RNAs) by RNA-dependent RNA polymerases (RdRPs) (not shown). Secondary siRNAs are incorporated in the nuclear Argonaute HRDE-1 and ultimately lead to H3K9me3 methylation, possibly other chromatin modifications (Chro Mod) and POL II repression in the germline. Resulting gametes carry a heritable signal, which may constitute secondary siRNAs, possibly in complex with HRDE-1 or other Argonautes, or chromatin modifications (Chro mod). This process is sometimes also referred to RNA-induced epigenetic silencing (RNAe). The Argonaute protein CSR-1 binds to a distinct set of 22G-RNAs and is thought to protect endogenous genes from aberrant silencing counteracting the effects of piRNAs by RNA-induced epigenetic gene activation (RNAa). (B) piRNA-mediated paramutation in the *D. melanogaster* germline. Conceptual representation of experimental approach and some of the results published by de Vanssay et al., (2012). A trans-silencing-proficient (TSE+) multi-copy *LacZ* cluster (T1) hemizygous over a balancer (Bal1; grey line) or a reporter transgene (not shown) in the maternal germline can convey silencing ability through a gamete, which carries the Bal1 genetic material but not the T1 cluster. When crossed to a male carrying a similar but TSE-deficient (TSE–) *LacZ* cluster (Bx2; represented with boxes), the maternal cytoplasm containing Aub:piRNA complexes conveys silencing capacity onto Bx2 in TSE assays for over 50 generations. The paramutated Bx2* allele generated in this way can further convey silencing ability onto a naive TSE- Bx2 allele when crossed in the same manner as described above, giving a second-order paramutated allele $Bx2^{*2}$. Short blue lines represent piRNAs, which are abundantly produced from T1. Paramutation turns Bx2* into a piRNA-producing locus. The small RNA sequences mapping to the respective locus are shown inside the nucleus for visualisation purposes only. Increase in piRNAs mapping to Bx2* upon paramutation is likely due to more efficient funneling of the Bx2 transcript into piRNA generation. Blue shading of cells is a visual representation of the ability of the *LacZ* cluster in this strain to induce trans-silencing effects in a standard TSE reporter assay and are not indicative of *LacZ* expression in a particular cell type.

the same element (Brennecke et al., 2008; Chambeyron et al., 2008). A lack of maternal piRNAs against the paternally contributed active transposable element is thus the cause of the dysgenic phenotype. Interestingly, this phenotype in the progeny can be rescued by aging of mothers lacking the transposable element: aged female ancestors are able to generate a sufficient amount of piRNAs

from heterochromatic remnants of the element via the secondary ping-pong cycle and can thereby provide protection to their progeny (Grentzinger et al., 2012).

Stable piRNA-mediated repression has also recently been described in *D. melanogaster* by de Vanssay et al. (2012). Here, silencing is active for over 50 generations and is reminiscent of

Box 1. Paramutation

The term paramutation was originally coined to describe heritable, non-genetic kernel colour variation in maize. It is defined as an 'interaction between alleles that leads to directed, heritable change at the locus with high frequency, and sometimes invariably, within the time span of a generation' (Brink, 1973). During this interaction *in trans*, a 'paramutagenic' allele induces a heritable epigenetic change in another 'paramutable' allele. This change persists even if the initiating paramutagenic allele is outcrossed and, moreover, the paramutated allele becomes paramutagenic itself. This should be distinguished from trans-silencing, which refers to repressive epigenetic interactions between two allelic or non-allelic loci while they are present in the same nucleus only. A plethora of examples for paramutation have been studied in plants where this mechanism of heritable silencing requires small RNAs and RNA-directed DNA methylation (Erhard and Hollick, 2011; Hollick, 2012). As plants do not have a developmentally dedicated germline, but rather generate meiotic cells from somatic tissues, heritable adaptation to environmental stresses is conceptually perhaps not so surprising (Bond and Baulcombe, 2014).

Box 2. Transgenerational epigenetic inheritance in mammals

Compelling evidence shows piRNA-mediated transgenerational effects in flies and nematodes. However, examples of transgenerational inheritance in mammals, such as the maternal epigenetic effects documented for the agouti coat phenotype in mice, are extremely rare (Daxinger and Whitelaw, 2012). The extensive reprogramming taking place in the zygote and germ cells of mammals argue against widespread propagation of epigenetic marks through generations. Whether environmental cues can induce bona fide transgenerational epigenetic change in mammals, particularly in humans, remains controversial. While *in utero* exposure to certain stresses affects progeny [e.g. seen in the Dutch famine winter studies where prenatal exposure of mothers leads to reduced birth length in offspring (Painter et al., 2008)], these effects are inter- rather than transgenerational: They are limited to F1 and F2 generations and reflect exposure of a foetus (and in maternal effect studies the germ cell lineage already present in that foetus) to stress (critically reviewed by Heard and Martienssen, 2014). The Överkalix study documented a true transgenerational effect where limited nutrition during childhood of paternal grandfathers affected mortality in grandsons (Pembrey et al., 2006). However, multigenerational data on humans are rare and often rely on participant interviews and retrospective data corrections to account for confounding effects. Recent studies in mice have started to control for factors such as cryptic genetic variation and parental provisioning, e.g. by cross-fostering. Ultimately, experimental design of utmost stringency will be required to determine whether there is indeed any evidence of Lamarckian inheritance in mammals.

paramutation – a silencing phenomenon that is well characterised in plants and that involves meiotically heritable changes in gene expression induced by transient interaction between allelic loci (Box 1) (Erhard and Hollick, 2011; Hollick, 2012). Using repeat clusters of P-element-derived *LacZ* transgenes, the authors were able to confer silencing capacity as assessed by reporter-based trans-silencing effect (TSE) assays onto a *LacZ* transgene cluster. This was achieved by exposing the 'naive', non-silencing cluster to cytoplasm of a strain carrying a similar cluster with strong TSE activity and did not require transmission of the actual silencing allele. (Fig. 3B). Silencing was dependent on the Piwi protein Aub and coincided with the generation of piRNAs matching to the transgene cluster (de Vanssay et al., 2012). Because the levels of sense and antisense transcript were unchanged when comparing the naive with the paramutated locus, the observed increase in piRNAs is likely based on more efficient funnelling of transcripts into the piRNA processing machinery either on the primary or the secondary, i.e. ping-pong, level.

The data discussed above provide evidence for piRNA-mediated transgenerational effects in flies and worms; however, signal transmission to the next generation is achieved by different means. In *Drosophila melanogaster*, all evidence indicates that maternally deposited piRNAs are the heritable agent. By contrast, the fact that transgenerational inheritance can become independent of the original piRNA trigger in *C. elegans* indicates that, at least during long-term maintenance of silencing, there are heritable signals other than maternally contributed Piwi:piRNA complexes in worms. Moreover, although there are some qualitative differences in trans-silencing and paramutation effects depending on parent of origin, there is, unlike in *D. melanogaster*, evidence for both maternal as well as paternal contribution to inheritance in nematodes (Shirayama et al., 2012; Wedeles et al., 2013).

It should furthermore be noted that the occurrence and stability of the silencing effects observed in *D. melanogaster* and *C. elegans* depend on the transgenes used: genomic location in allelic versus non-allelic loci and copy number of transgenes leads to varying levels of paramutability in *D. melanogaster* (de Vanssay et al., 2012), and orientation as well as length of tags influences *ab initio* likelihood for PRG-1-dependent transgene silencing in *C. elegans* (Shirayama et al., 2012). Interestingly, recent evidence indicates that *C. elegans* has evolved a small RNA-based anti-silencing mechanism that provides a signature of 'self' that opposes the piRNA-mediated signature of

'non-self' (Seth et al., 2013; Wedeles et al., 2013). In this model, small RNAs bound to the Argonaute CSR-1 recognise, but do not downregulate, germline-expressed genes. Instead, targeting by CSR-1 serves as a molecular marker of 'self' and counteracts silencing by other small RNA pathways, including the piRNA pathway (Fig. 3A) (Shirayama et al., 2012). The extent of 'self-ness' of a transgene may therefore determine whether it is silenced by the piRNA pathway. Whether the tremendous targeting potential of the thousands of individual 21U-RNAs, all capable of recognising targets with non-perfect sequence complementarity (Bagijn et al., 2012), can be employed to recognise invading repeat elements that do not carry the self signature remains to be experimentally validated.

Although the study of small RNA-mediated transgenerational effects in insects and nematodes is still in its infancy, its importance is underlined by some of the phenotypes observed. In *C. elegans*, the loss of *prg-1* leads to transgenerational germline mortality (*mrt*) (Simon et al., 2014). Briefly, unlike Piwi mutant animals in other organisms, *C. elegans prg-1* mutants are not immediately sterile and, when freshly outcrossed, display relatively mild fertility defects while still producing fertile offspring. However, subsequent generations of *prg-1* mutants become progressively sterile. This effect is likely epigenetic as no genetic lesions, e.g. through transposon mobilisation, consistent with the loss of germline fertility were observed.

Despite the findings detailed above that clearly document piRNA-mediated transgenerational silencing in *C. elegans* and *D. melanogaster*, inter- and transgenerational effects of the Piwi/piRNA pathway are not very well studied in vertebrates. Analysis of piRNA populations in zebrafish hybrids show evidence for maternal effects on the ratio of sense and antisense piRNAs in the progeny (Kaaij et al., 2013). Despite similarities between the fly and zebrafish piRNA pathways, including presence of a ping-pong loop and observed fertility phenotypes (Houwing et al., 2007, 2008), further investigation will be needed to determine the extent of inheritance in this system. Examples of transgenerational inheritance in mammals are also extremely

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rare, and whether transgenerational inheritance truly occurs in mammals, particularly in humans, remains controversial (Box 2).

Conclusions

In recent years, remarkable progress has been made in understanding the piRNA pathway in a variety of organisms. With the identification of a large number of proteins involved in piRNA biogenesis in different systems, we can now progress to elucidating the varying mechanistic processes underlying piRNA production and function.

In addition, the piRNA targeting mechanism provides further opportunity for investigation. Nuclear small RNA-mediated silencing has been a topic of great interest in recent years and many advances have been made in this field. TGS or co-transcriptional gene silencing by RNAi has been particularly well studied using the fungus model *S. pombe* (for a review, see Creamer and Partridge, 2011). Work on RNA-directed DNA methylation in plants has also yielded insights into small RNA-mediated silencing in higher organisms (reviewed by Zhang and Zhu, 2011). Recent findings describing the role of transcriptional regulation of transposons downstream of piRNAs for the first time provide a paradigm for studying transcriptional regulation by small RNAs in animals, and the use of *C. elegans* and *D. melanogaster* as simple model systems promises important new insights into this process. For example, potential interactions between nuclear Argonaute:small RNA complexes and pre-mRNAs or DNA, and the order of events leading to POL II repression and chromatin modification, can now be probed experimentally. Reports of piRNA target repression by mRNA deadenylation also raises the possibility that post-transcriptional silencing downstream of piRNAs may be more varied than previously anticipated (Gou et al., 2014; Rouget et al., 2010). Further investigation into this previously uncharacterised cytoplasmic piRNA-mediated silencing mode should provide tremendously exciting results.

Strikingly, results from studies on transgene silencing have shown that piRNA targets can be stably silenced across generations. When and how such heritable silencing is initiated is another exciting avenue for investigation, in particular as reports of bona fide paramutation in animals remain rare (reviewed by Heard and Martienssen, 2014). Although flies and nematodes are particularly amenable to studying effects that provide memory across several generations, one report has remarkably also revealed involvement of piRNAs in paternal imprinting of a single mouse locus (Watanabe et al., 2011b).

Understanding the piRNA target spectrum provides further challenges, in particular in systems where piRNAs are not perfectly complementary to transposable elements. Indeed, such mismatch targeting occurs in *C. elegans* and this, given the sheer amount of unique piRNAs, raises the issue of how aberrant gene repression can be avoided. In the nematode, a protective small RNA system capable of recognising 'self' may be one of the mechanisms protecting germline transcripts (Seth et al., 2013; Wedeles et al., 2013). The extent of mismatch targeting in *D. melanogaster* and mice is less clear, although several examples of imperfect complementarity have been published for both organisms (Gou et al., 2014; Rouget et al., 2010; Saito et al., 2009). Further insights into the spatial and temporal compartmentalisation of the piRNA machinery will be required to fully appreciate how correct targeting can be achieved.

One final important new aspect of piRNA silencing, which could not be discussed here due to space limitations, is the role of piRNAs outside the germline. Although the presence of Piwi and piRNAs is well established in ovarian somatic tissues, the expression of Piwi in salivary glands and throughout different developmental stages in

D. melanogaster has also been documented (Brower-Toland et al., 2007). Strikingly, differential expression of Aub and Ago3 proteins in the fly mushroom body, a brain structure involved in olfactory memory, leads to relaxed transposon repression in a subset of neurons (Perrat et al., 2013). It will be fascinating to see whether the resulting genetic heterogeneity in these cells may be associated with learning processes. In the mouse, MIWI:piRNA complexes have also been detected in the hippocampus (Lee et al., 2011). Furthermore, piRNAs are present in the nervous system and other somatic tissues of the sea slug *Aplysia*, where they positively regulate long-term synaptic facilitation (Rajasethupathy et al., 2012). Future in-depth studies of these and other examples will greatly contribute to our understanding of the significant roles played by Piwi proteins and their associated small RNAs. We look forward to these and many other exciting findings yet to be made in the piRNA field.

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