Trends in Genetics

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Review

Functions of PIWI Proteins in Gene Regulation: New Arrows Added to the piRNA Quiver

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Piwi-interacting RNAs (piRNAs) and PIWI proteins play key functions in a wide range of biological and developmental processes through the regulation of cellular mRNAs, in addition to their role in transposable element (TE) repression. Evolutionary studies indicate that these PIWI functions in mRNA regulatory programs, occurring in both germ and somatic cells, are ancestral. Recent advances have widely expanded our understanding of these functions of PIWI proteins, identifying new mechanisms of action and strengthening their importance through their conservation in distant species. In this review, we discuss the latest findings regarding piRNA/PIWI-dependent mRNA decay in germ cells and during the maternal-to-zygotic transition in embryos combined with new modes of action of PIWI proteins in mRNA stabilization and translational activation and piRNA-independent roles of PIWI proteins in cancer.

Highlights

piRNAs and PIWI proteins have widespread functions in cellular mRNA regulation programs.

mRNA regulation by PIWI proteins is conserved in both the molecular mechanisms involved and the biological functions achieved, despite the lack of piRNA sequence conservation.

piRNAs and PIWI proteins are implicated in maternal mRNA decay during the maternal-to-zygotic transition in distant species.

PIWI proteins activate the translation of target mRNAs by direct recruitment of the eIF3 translation initiation factor and poly(A) binding protein.

PIWIL1 regulates protein homeostasis by a piRNA-independent mechanism in cancer cells.

The piRNA Pathway and the Range of Its Functions

piRNAs (see Glossary) are a class of small noncoding RNAs expressed mainly in animal germlines. They are primarily a defense mechanism for the germline genome against **TE** mobilization and invasion (reviewed in [1–4]). piRNAs are 21–32 nucleotides in length and associate with specific ARGONAUTE proteins of the PIWI clade: MIWI, MILI, and MIWI2 in the mouse; PRG-1 in *Caenorhabditis elegans*; and Piwi, Argonaute 3 (Ago3), and Aubergine (Aub) in *Drosophila*. In mice and *Drosophila*, a large proportion of piRNAs originate from arrays of defective TEs assembled in loci that have originally been called 'piRNA clusters'. These clusters adapt by the insertion of newly invading transposons, producing new piRNAs that repress cognate active TEs [5,6]. The biogenesis of piRNAs is tightly intertwined with their role as TE silencers. A unified model of piRNA biogenesis has recently been reported, which explains piRNA production in most species, indicating evolutionary conservation for about 800 million years [7] (Box 1).

Beside this well-documented role in TE repression, functions of **PIWI proteins** and piRNAs in the regulation of gene expression have also been described early. Cellular mRNA regulation by piRNAs and PIWI proteins contribute to various cellular and developmental processes, such as embryonic patterning in *Drosophila* and spermatogenesis in the mouse, through the decay of large numbers of mRNAs [8–11]. PIWI proteins were first discovered based on their conserved functions in germline stem cell self-renewal [12], and these functions were shown more recently to involve cellular mRNA regulation [13,14]. These proteins are found in a variety of stem cells, including somatic stem cells, and are required for stem cell stemness in primitive species such as planaria, which are known to be associated with high regenerative capacity (reviewed in [15]). In these species, a large proportion of piRNAs are produced from genes (genic piRNAs) suggesting functions of the piRNA pathway in gene regulation. Genic piRNAs are abundant in many species, consistent with conserved functions of PIWI proteins in the post-transcriptional regulation of developmental programs, with recent evolutionary studies proposing that these PIWI/piRNA functions could be ancestral [16,17].

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Box 1. piRNA Biogenesis and Function in TE Repression

The unified model of piRNA biogenesis proposes that piRNA production involves two interconnected mechanisms: phasing and ping-pong amplification. Interestingly, these two mechanisms are physically separated in the cell, ping-pong taking place in nuage comprising membraneless ribonucleoprotein granules localized at the periphery of germ cell nuclei and phasing occurring at the mitochondrial outer membrane [69–73]. First, piRNA cluster genes [74] are transcribed into long noncoding transcripts by RNA polymerase II via specific transcription factors or chromatin regulators [2,3] (Figure 1). The piRNA precursor transcript is then exported out of the nucleus in the nuage, with a specific export complex containing nuclear export factor 3 (Nxf3) in *Drosophila* [75,76]. There, *Drosophila* Ago3 associated with a maternally inherited piRNA recognizes and cleaves a complementary piRNA precursor transcript, generating a 5′ monophosphorylated RNA that interacts with Aub. New piRNAs can then be produced by either ping-pong or phasing. In the ping-pong cycle, the piRNA precursor is cleaved again further downstream by Ago3 to generate the piRNA 3′ end. This new piRNA loaded into Aub targets and drives the slicing of complementary TE transcripts, generating piRNAs that are loaded into Ago3. In turn, piRNA-loaded Ago3 binds to and slices piRNA precursor transcripts to generate piRNAs that associate with Aub. This reciprocal cleavage by Aub and Ago3 results in the production of complementary piRNAs with an overlap of ten nucleotides, a signature of the ping-pong amplification cycle (reviewed in [1]). For piRNA generation by phasing, the cleaved piRNA precursor transcript interacting with Aub travels with the RNA helicase Armitage to the mitochondrial outer membrane [70]. The endonuclease Zucchini (MitoPLD in the mouse) directed by Aub cleaves the piRNA precursor transcript generating the 3′ end of a new piRNA loaded into Aub. The rest of the piRNA precursor transcript is repeatedly bound at its 5′ end by Piwi and cleaved by Piwi-guided Zucchini, generating phased production of Piwi-loaded piRNAs toward the 3′ end of the piRNA precursor transcript [77–81]. While the phased piRNA production generates *de novo* piRNAs increasing piRNA diversity, the ping-pong amplification cycle increases the pool of pre-existing piRNAs.

In addition to this post-transcriptional silencing of TEs, piRNAs repress TEs transcriptionally. piRNAs produced by phasing and loaded into nuclear PIWI proteins (Piwi in *Drosophila*, MIWI2 in the mouse) travel to the nucleus where they interact with TE nascent transcripts. PIWI proteins then establish repression through a complex network of chromatin remodelers [82–87].

In this review, we focus on recent advances made in uncovering both the biological functions of PIWI proteins and their mechanisms of action. While PIWI proteins act mostly by mRNA **slicing (or cleavage)** through their endonuclease activity, on base pairing of target mRNA with piRNAs, novel mechanisms of action of PIWI proteins in mRNA stabilization and translational activation have been identified. Recent studies have also revealed the conserved function of the piRNA pathway in maternal mRNA decay during the maternal-to-zygotic transition and established piRNA-independent roles of PIWI proteins in tumorigenesis and metastasis. These new findings largely expand the breadth of piRNA and PIWI protein functions in healthy development and pathological processes, through a variety of molecular mechanisms.

Degradation of Cellular mRNAs by piRNAs and PIWI Proteins

Since PIWI proteins cleave TE mRNAs, they can be easily considered able to degrade cellular mRNAs. This would require the insertion of TE-related sequences into mRNA untranslated regions (UTRs), the production of piRNAs from genes with similar sequence (e.g., pseudogenes), or low-complementarity-based targeting of mRNAs with piRNAs produced from TEs or repeated sequences. These different modes of cellular mRNA recognition by piRNAs have indeed been described in various contexts and species (reviewed in [15]).

One such example has been the cause of experimental troubles for decades with *UAS* transgenes in the *Drosophila* germline but has only recently been identified as ensuing from piRNA-mediated regulation (Box 2). Beyond this technical example, piRNA-mediated degradation of cellular mRNAs regulates a whole cohort of genes essential for various developmental programs. Mouse spermatogenesis is one of those. In mouse testes, whereas fetal piRNAs are produced from TE sequences and regulate TEs, pachytene piRNAs that are produced in adults at the pachytene stage of meiosis are unrelated to TEs. Pachytene piRNAs are mostly loaded into MIWI, a cytoplasmic PIWI protein. Crosslinking and immunoprecipitation (CLIP) assays of

Glossary

Deadenylation: the process of poly (A) tail shortening where adenosine nucleosides are removed from the 3′ end of mRNAs by a poly(A)-specific 3′ exonuclease. The CCR4-NOT complex that is conserved from yeast to human is the predominant deadenylase. It comprises eight proteins including two deadenylases, CCR4 and CAF1/POP2. Deadenylation triggers full mRNA decay.
Germ plasm: specialized cytoplasm present at the posterior pole of *Drosophila* oocytes and embryos. It comprises germ granules in which germ cell mRNAs are stored and tightly regulated to participate in the formation of posterior embryonic structures and germ cell development.
Maternal-to-zygotic transition (MZT): period at the beginning of embryonic development during which the control of development switches from the maternal to the zygotic genome; maternal mRNAs are massively degraded and transcription of the zygotic genome is activated.
Ping-pong: mechanism of piRNA biogenesis leading to piRNA amplification and achieved through reciprocal cleavage of piRNA-targeted transcripts by PIWI proteins. Due to cleavage by PIWI proteins after nucleotide 10 of piRNAs, ping-pong produces piRNA pairs that are complementary over ten nucleotides.
PIWI-interacting RNAs (piRNAs): small RNAs defined by their ability to associate with PIWI proteins. They are 21–32 nucleotides long depending on species and display 2′-*O*-methylation at their 3′ end.
PIWI proteins: *P*-element-induced wimpy testis (Piwi), the first PIWI protein, was identified genetically in *Drosophila* from its role in germline stem cell self-renewal. PIWI proteins form a specific family among ARGONAUTE proteins, based on phylogenetic analyses.
Polyadenylation: the process of poly (A) tail lengthening, where a poly (A) polymerase adds adenosines to the 3′ end of the mRNA. Cytoplasmic polyadenylation involves specific proteins different from those required for nuclear polyadenylation.
Seed: the seed sequence has been defined for miRNAs and corresponds to nucleotides 2–7 of the miRNA, where perfect complementarity with the target mRNA is required for regulation. Additional complementarity in the

Box 2. Repression of *UAS* Transgenes by *Hsp70* piRNAs in *Drosophila* Germ Cells

In *Drosophila* developmental genetics, the *UAS/Gal4* inducible expression system set up in the 1990s has been unavoidable [88]. This yeast-derived system involves the transcriptional activator Gal4 and its binding sequence *UAS* upstream of a minimal *Hsp70* promoter. Localized Gal4 expression allows spatial and temporal expression of a cDNA cloned downstream of the *UAS-Hsp70* promoter. It soon became evident that the system was working in somatic tissues but not in germ cells [89], although the reason was not understood. A recent study investigated this question and showed that the piRNA pathway is responsible for the *Hsp70*-driven repression of *UAS* transgenes in the germline [90]. The *Hsp70* gene clusters produce germline-specific piRNAs that target the *Hsp70* sequence present in *UAS* expression vectors. Furthermore, the presence in the genome of a *Hsp70*-containing transgene stimulates the cleavage of endogenous *Hsp70* transcripts, increasing the amount of *Hsp70* piRNAs [91]. Reducing *Hsp70* regions in new *UAS* vectors results in high expression in germ cells, clearly demonstrating that piRNA base pairing with a cellular mRNA can efficiently induce its repression [90].

MIWI revealed its binding to a large repertoire of cellular mRNAs, pointing to a role in post-transcriptional regulation of gene expression [11,18]. Different modes of MIWI-dependent mRNA decay have been reported. piRNAs produced from ancient TEs target the same TEs inserted into long noncoding RNAs or mRNA 3′UTRs, leading to mRNA cleavage by MIWI slicer activity [19]. piRNAs produced from pseudogenes present in piRNA clusters were also proposed to repress their cognate mRNAs [19]. However, a recent evolutionary study revealed a lack of functional constraint on pseudogene-derived piRNA targeting, suggesting a minimal contribution of pseudogene piRNAs in mRNA regulation [20]. Finally, incomplete base pairing of mRNAs with pachytene piRNAs was shown to lead to either mRNA slicing by MIWI, generating genic piRNAs by **ping-pong**, or mRNA decay by **deadenylation** through the recruitment by MIWI of the CAF1 deadenylase [9,11,21] (Figure 1A, Key Figure). These two outcomes depend on the level of base paring between piRNAs and their target mRNAs and on the formation of the MIWI–CAF1 complex occurring specifically in late spermatids. Importantly, both piRNA cluster gain-of-function and lack-of-function approaches showed that defects in mRNA regulation by pachytene piRNAs lead to mouse male sterility, demonstrating the key role of this regulatory mechanism in sperm maturation [21,22]. Removal of piRNAs produced from different piRNA clusters does not systematically lead to phenotypic defects, possibly due to redundancy. In addition, a small number of the piRNAs in a piRNA cluster are responsible for mRNA cleavage and sperm defects, consistent with the high level of base pairing required for slicing [21].

A recent study in the planarian *Schmidtea mediterranea* revealed that one of the three PIWI proteins also targets a large repertoire of mRNAs, leading either to their decay through ping-pong or to binding without degradation [23]. Again, mRNA slicing by PIWI depends on the level of piRNA complementarity: strong base pairing, particularly across nucleotides 10 to 11 leads to mRNA cleavage, whereas uncleaved mRNAs show a lack of base pairing at nucleotides 10 and 11 and after nucleotide 16 [23].

The piRNA pathway is rather different in *C. elegans* in that mRNA targeting by piRNAs (called 21U-RNAs) induces the production from target mRNAs of secondary antisense small RNAs (called 22G-RNAs) that loaded, into other ARGONAUTE proteins (WAGOs or CSR-1), are the effectors of mRNA target regulation (reviewed in [3]). In this species, piRNAs target most germline mRNAs through partial complementarity similar to miRNA **seed**-based interactions [24,25]. This leads to transcriptome-wide mRNA regulation by piRNAs, whereas their role in regulating TEs remains modest [26,27].

Strikingly, the silencing of a specific *C. elegans* mRNA, *xol-1* by an X-linked piRNA plays a key role in sex determination and dosage compensation [28]. This piRNA developmental function is remarkable because it appears to be conserved in a distant species of nematode, *Caenorhabditis*

miRNA 3′ region is also generally necessary to achieve regulation.

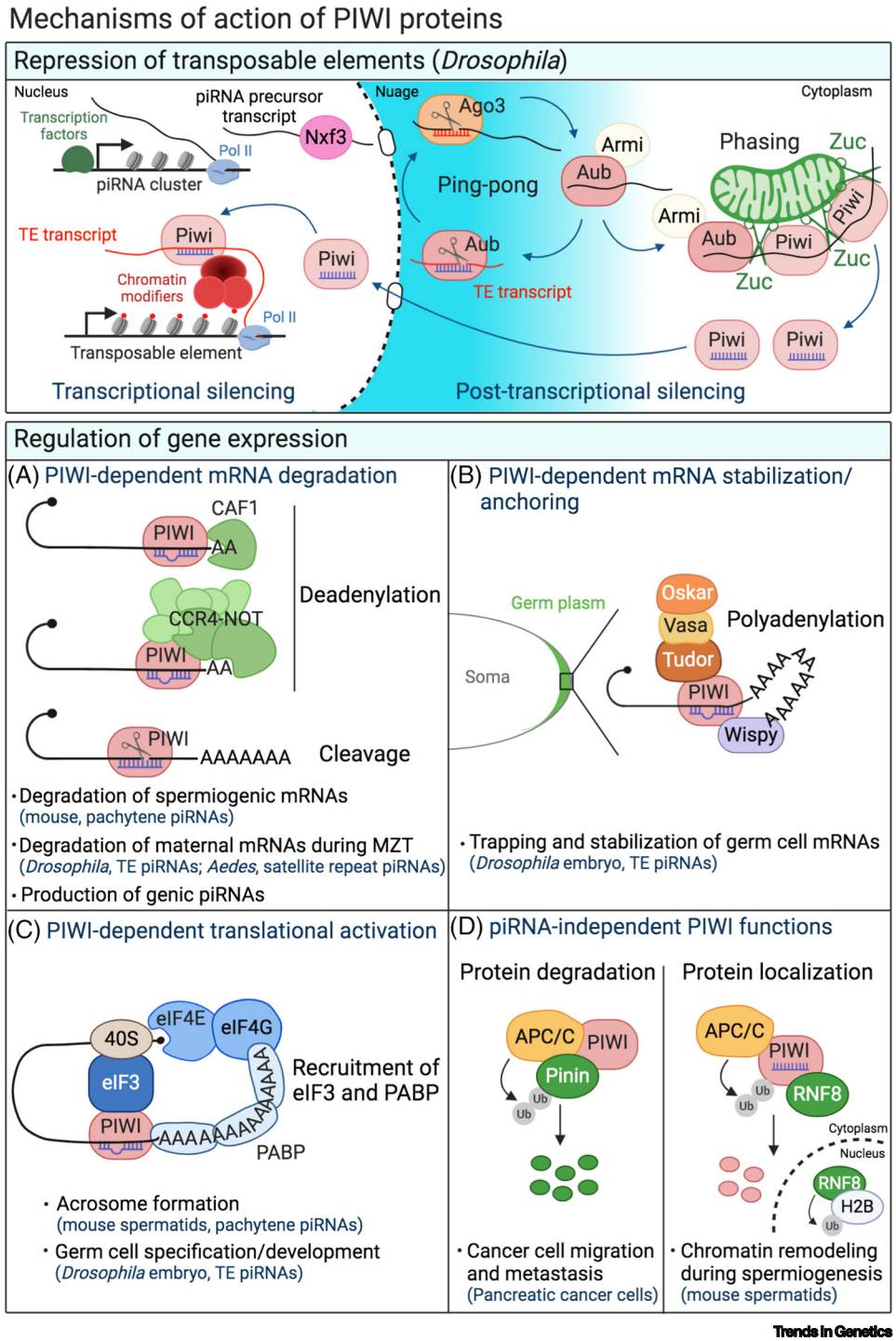
Slicing (or cleavage): ARGONAUTE-mediated cleavage of target mRNAs, occurring between nucleotides 10 and 11 from the 5′ end of the complementary piRNA.

Spermiogenesis: complex differentiation program at the end of spermatogenesis where spermatids differentiate and mature into spermatozoa. This program includes chromatin compaction leading to transcription repression and thus depends, after this step, on the regulation of stored mRNAs.

Transposable elements (TEs): DNA sequences that have the ability to move into a genome. They lead to various types of genomic alterations and rearrangements that may be deleterious. However, they also increase genome diversity and thus play a major role in genome dynamics and evolution.

Key Figure

The Different Mechanisms of Action of PIWI Proteins



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briggsae, and, more surprisingly, similar regulation also occurs in the silk worm, *Bombyx mori*, that is separated from nematodes by 700 million years. In *B. mori*, a female-specific piRNA represses, through ping-pong, the *Masculinizer* mRNA that controls sex determination and dosage compensation in males [29]. This piRNA-mediated regulation is essential for female differentiation. Therefore, mRNA degradation by piRNAs has crucial functions in various developmental processes related to sexual reproduction such as germ cell development, sex determination, and dosage compensation.

Another important role of piRNA-mediated mRNA decay has been recently reported in reproductive isolation between species. In *Drosophila* testes, the most abundant piRNAs are unrelated to TEs and originate from repeated sequences: the *Suppressor of Stellate* [*Su(Ste)*] and *AT-chX* loci located on the Y and X chromosome, respectively [30–33]. Deletion of the *Su(Ste)* locus leads to a significant increase of *Stellate* (*Ste*) mRNA and protein levels, abnormal spermatocyte development, and male sterility, thus revealing the biological importance of *Ste* mRNA repression by *Su(Ste)* piRNAs [30,34]. However, *Ste* and *Su(Ste)* are not conserved in related *Drosophila* species. By contrast, *AT-chX* piRNAs show sequence similarity with *vasa* mRNA that encodes a key determinant of germ cell specification [33]. *vasa* is not highly regulated by *AT-chX* piRNAs, however, due to low complementarity. Unexpectedly, *AT-chX* piRNAs show stronger base pairing with *vasa* mRNA from a closely related species, *Drosophila mauritiana* [31]. Interspecies crosses between *Drosophila melanogaster* and *D. mauritiana* produce males with reduced numbers of germ cells and meiotic failure, resulting in part from repression of *D. mauritiana vasa* mRNA by *AT-chX* piRNAs and derepression of *Ste* mRNA due to the lack of *Su(Ste)* piRNAs [31]. Therefore, the repression of cellular mRNAs by piRNAs and PIWI proteins not only is involved in germline development and sexual reproduction, but also appears to contribute to reproductive isolation between closely related species. Intriguingly, a recent study revealed the high divergence of pachytene piRNAs between placental mammals and even within the human population, and proposed that piRNA diversity might act in reproductive isolation [35].

Figure 1. Top panel: Transcriptional (left) and post-transcriptional (right) repression of transposable elements (TEs) by piwi-interacting RNAs (piRNAs) and PIWI proteins. piRNA precursor transcripts are produced by RNA polymerase II (Pol II) from piRNA cluster genes and exported to the nuage (blue). Here, piRNA-loaded Argonaute 3 (Ago3) slices a complementary piRNA precursor transcript, generating a cleaved RNA with a new 5′ end that is bound by Aubergine (Aub). This RNA fragment is cleaved into piRNAs via phasing, by the Zucchini endonuclease (Zuc) at the mitochondrial outer membrane, or via ping-pong in the nuage. In the second part of the ping-pong cycle, piRNA-loaded Aub targets and cleaves a complementary TE mRNA, generating piRNAs loaded into Ago3. Reciprocal cleavage by Aub/Ago3 contributes to TE post-transcriptional repression and amplification of the piRNA pool. Piwi loaded with piRNAs during phasing translocates to the nucleus, associates with TE nascent transcripts and recruits chromatin remodelers, inducing transcriptional silencing through histone modifications (red dots). Bottom panel: Molecular mechanisms of PIWI and piRNA functions in the regulation of gene expression. (A) PIWI-dependent cellular mRNA degradation. PIWI proteins promote mRNA decay through either the recruitment of deadenylation factors – CAF1 deadenylase or the CCR4-NOT complex – or mRNA cleavage, depending on the level of base pairing between piRNAs and target mRNAs. (B) PIWI-dependent mRNA stabilization. In *Drosophila* oocytes and early embryos, Aub binds germ cell mRNAs through piRNA base pairing. Whereas in the soma Aub binding leads to the decay of these mRNAs (A), in the germ plasm (green) Aub accumulates through direct interaction with Tudor and contributes to the localization of the same mRNAs. Aub directly recruits the cytoplasmic poly(A) polymerase Wispy, leading to mRNA polyadenylation and stabilization in the germ plasm. Components of germ granules, such as Tudor, Vasa, and Oskar, are likely to participate in the switch in Aub function from mRNA decay to stabilization between soma and germ plasm. (C) PIWI-dependent translational activation. PIWI proteins directly recruit the eIF3 translation initiation factor and the poly(A) binding protein (PABP), thus contributing to mRNA 5′–3′ end interactions and leading to translational activation. (D) piRNA-independent functions of PIWI proteins. PIWI proteins interact with the anaphase-promoting complex/cyclosome (APC/C) complex acting as either an activator (in cancer cells) or a target (in spermatids) of APC/C-mediated protein degradation. Left panel: In cancer cells, unloaded PIWI bound to APC/C triggers the ubiquitylation and degradation of the tumor suppressor Pinin, facilitating cancer cell migration and metastasis. Right panel: In elongated mouse spermatids, timely APC/C-dependent degradation of PIWI allows the release and nuclear translocation of the ubiquitin ligase RNF8, essential for histone exchange with protamines. Abbreviation: MZT, maternal-to-zygotic transition.

A Key Role of piRNAs and PIWI Proteins in the Maternal-to-Zygotic Transition

In most species, the zygotic genome is silent in the early embryo; therefore, maternally deposited mRNAs orchestrate the first steps of development. Maternal mRNAs are then progressively eliminated and the zygotic genome starts to be expressed, taking control of development in a process called the **maternal-to-zygotic transition (MZT)**. Maternal mRNA decay is accomplished by two pathways that are maternally and zygotically encoded (reviewed in [36]). Recent data have highlighted the conservation of piRNA and PIWI protein function in maternal mRNA decay during the MZT (Figure 2).

The role of piRNAs in maternal mRNA degradation in *Drosophila* has been the first clear example of cellular mRNA regulation by piRNAs with a role in development. *nanos* (*nos*) mRNA is one of the maternal mRNAs that is degraded in the early *Drosophila* embryo. It encodes a key determinant of posterior patterning and germ cell development. *nos* mRNA regulation involves a complex interplay between degradation, stabilization, and translational control allowing the distribution of Nos protein as a gradient emanating from the posterior pole of the embryo. *nos* mRNA is initially present in the whole embryo with about 4% being localized and stabilized at the posterior pole in the so-called **germ plasm** [37,38]. The rest of the *nos* mRNA in the somatic part of the embryo is degraded by both the maternal and zygotic pathways of mRNA decay [39]. The cytoplasmic PIWI protein Aub loaded with piRNAs is provided maternally to the embryo and contributes to *nos* mRNA decay; it is therefore a component of the maternal pathway of mRNA decay [10] (Figure 2). Aub cooperates with two other components of this pathway, the RNA-binding protein Smaug and the CCR4-NOT deadenylation complex. *nos* mRNA decay by Aub involves not its endonuclease activity, but rather deadenylation by CCR4-NOT. piRNAs produced from two TEs, *roo* and *412*, target the *nos* 3′ UTR with partial base pairing. piRNA target sites in the *nos* sequence, as well as the piRNAs themselves, are necessary for *nos* mRNA decay and embryonic development, revealing an intriguing role for TEs in this developmental process [10]. Aub CLIP assays in *Drosophila* embryos have identified more than 600 mRNAs bound by Aub in a piRNA-dependent manner [8]. Aub is involved in the decay of germ cell mRNAs that, similar to *nos*, are unstable in the soma and accumulate in the germ plasm to participate in germ cell development. Interestingly, some of these mRNAs produce genic piRNAs with ping-pong signatures, indicating cleavage by Aub. Thus, Aub degrades maternal mRNAs by two mechanisms: cleavage and recruitment of the CCR4-NOT complex [8,10].

A recent study revealed a similar piRNA-mediated maternal mRNA decay in the mosquito *Aedes aegypti* [40]. In this system, piRNAs are not produced from TE sequences but from satellite repeats that have been conserved in mosquitos for more than 200 million years. Two piRNAs derived from satellite repeats associate with the PIWI protein Piwi4 and degrade maternal mRNAs in mosquito embryos. Injection of oligonucleotides antisense to piRNAs in early embryos leads to early developmental arrest and increased levels of target mRNAs. These two piRNAs are not maternally deposited but expressed from the zygotic genome. Therefore, in *A. aegypti*, piRNAs are part of the zygotic pathway of maternal mRNA decay. These piRNAs act similarly to specific miRNAs in *Drosophila* and zebrafish, which are also expressed zygotically and target a large number of maternal mRNAs to induce their decay during the MZT [41,42]. Analyses of the base-pairing requirement of mosquito piRNAs have shown the implication of the seed and additional base pairing either directly following the seed or in the 3′ part of the piRNA. However, complementarity at the potential cleavage site (positions 10/11 of the piRNA) was dispensable, indicating a cleavage-independent mode of mRNA decay [40].

Remarkably, in *C. elegans* a role for 22G-RNAs produced from mRNA targeting by piRNAs has also been reported in maternal mRNA decay during the MZT. 22G-RNAs loaded into the Argonaute protein CSR-1 are provided maternally to the embryo and initiate the degradation of complementary mRNAs through CSR-1 cleavage activity [43].

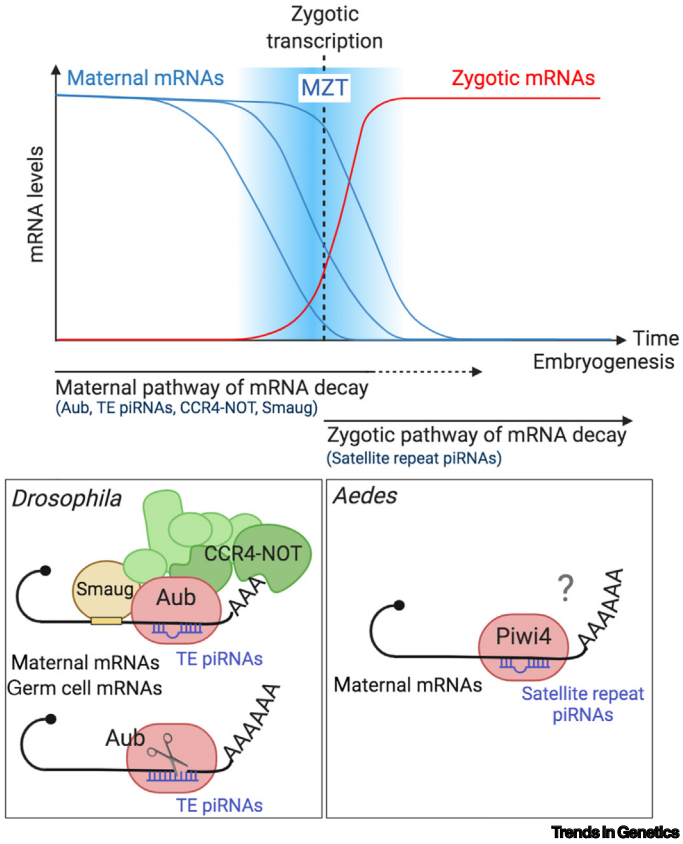

Figure 2. Function of Piwi-Interacting RNAs (piRNAs) and PIWI Proteins during the Maternal-to-Zygotic Transition (MZT). Top panel: Profiles of maternal unstable mRNAs (blue lines) and of zygotic mRNAs (red line) are shown. Both maternal and zygotic factors contribute to maternal mRNA decay leading to sequential degradation. The broken line indicates the start of the bulk of zygotic transcription. Bottom panels: In *Drosophila*, Aubergine (Aub) and transposable element (TE) piRNAs are part of the maternal factors required for maternal mRNA decay, together with Smaug and the CCR4-NOT complex. Aub promotes maternal mRNA clearance by deadenylation or cleavage. In *Aedes*, Piwi4 is provided maternally, whereas satellite repeat piRNAs that target maternal mRNAs are expressed by the zygotic genome. Therefore, Piwi4/piRNAs are part of the zygotic pathway of mRNA decay. Satellite repeat piRNAs target maternal mRNAs with loose base pairing and contribute to their clearance by an unknown mechanism (indicated by a question mark).

These studies point to a widespread role among animals of the piRNA pathway in the MZT, a key developmental program driving early embryogenesis. How did piRNAs become players in this developmental process? Various mechanisms contribute to mRNA clearance during the MZT [36]. As an efficient system to degrade mRNAs, the piRNA pathway might have been coopted to provide an additional level of mRNA decay during the MZT and increase the robustness of the process.

piRNAs and PIWI Proteins in Stabilization of Cellular mRNAs

Low base pairing between piRNAs and their target mRNAs does not allow slicing by PIWI proteins, but rather the recruitment of regulators such as the CCR4-NOT complex [9,10]. Recruitment of different regulators would mediate various outcomes in mRNA regulation, including positive regulation.

In the early *Drosophila* embryo, Aub and piRNAs are part of the developmental program leading to germ cell mRNA decay in the soma [8], but Aub is also a core component of germ granules that

constitute the germ plasm, where these mRNAs are stabilized [38,44]. These conflicting data were analyzed using *nos* mRNA as a paradigm. Aub and piRNA target sites in *nos* 3′UTR were shown to be required for *nos* mRNA stabilization in the germ plasm [45]. At the mechanistic level, Aub directly interacts with the germline-specific cytoplasmic poly(A) polymerase Wispy that accumulates in the germ plasm, inducing mRNA **polyadenylation** and stabilization (Figure 1B). In line with this positive role of Aub and piRNAs, Aub was reported to be involved in anchoring germ cell mRNAs to the germ plasm during late oogenesis [46]. The molecular basis of the switch in Aub function between soma and germ plasm remains unknown, although additional factors – in particular specific components of the germ plasm such as Oskar or Tudor, a direct Aub interactor – are likely to be involved.

Germ granules are conserved structures in germ cells across metazoans. They contain mRNAs required for germline specification and development as well as PIWI proteins, suggesting that this role of PIWI proteins in mRNA stabilization and storage in germ granules might be conserved. Consistent with this, recent studies in *C. elegans* have revealed that piRNA/PIWI-mediated mRNA accumulation in germ granules prevents their silencing [47,48].

Translational Activation: A New Mode of Action of piRNAs and PIWI Proteins

Early reports pointed toward a role for PIWI proteins in the regulation of translation. Both MIWI and MILI were found to associate at different steps of mouse spermatogenesis with the mRNA cap-binding complex that is required for translation activation [49,50]. More recently, Aub was shown to regulate protein levels without affecting the cognate mRNA poly(A) tail length in germline stem cells of *Drosophila* ovaries, indicating a potential role in translational regulation [14]. A link was also reported between Aub and eIF4 translation initiation factors in *Drosophila* cultured cells [13].

Two recent studies have established the role of PIWI proteins in translational activation and revealed the underlying mechanisms in mouse spermatids and *Drosophila* embryos [51,52]. On the analysis of pachytene piRNA-induced mRNA decay in a spermatocyte-derived cell line, an increase in protein levels without an effect on mRNA levels was found instead for specific piRNAs, suggesting a role in translational activation [51]. This positive effect was shown to require MIWI, and a combination of CLIP assays and ribosome profiling identified a large group of mRNAs potentially regulated by MIWI. AU-rich elements (AREs) in 3′UTRs were found to be the common element in these mRNAs, and the insertion of an ARE was sufficient to switch the regulation of an mRNA from decay to translational activation. HuR protein, known to bind AREs, associated with MIWI-activated mRNAs and contributed to translational activation (Figures 1C and 3). Importantly, many mRNAs subject to MIWI-dependent translational activation are involved in spermatid development, and the regulation of two such mRNAs by MIWI was demonstrated to play a crucial role in acrosome formation [51].

In the *Drosophila* embryo, translational regulation of germ cell mRNAs is intimately linked to their stability. These mRNAs are translationally repressed and unstable in the soma, whereas they are stabilized and translated in the germ plasm. Consistent with this, the *nos* translational repressor complex contains components involved in both translational repression and mRNA decay, such as Smaug and the CCR4-NOT deadenylation complex [53]. As Aub in involved in *nos* mRNA stabilization in the germ plasm [45], its potential role in translational activation was analyzed. Because Oskar protein drives germ plasm assembly at the posterior pole of embryos, overexpression of Oskar in the whole embryo was used to mimic the germ plasm. In this context, Aub is indispensable for *nos* mRNA translation [52]. piRNAs are also involved in this Aub function, as Nos protein levels depend on both piRNA

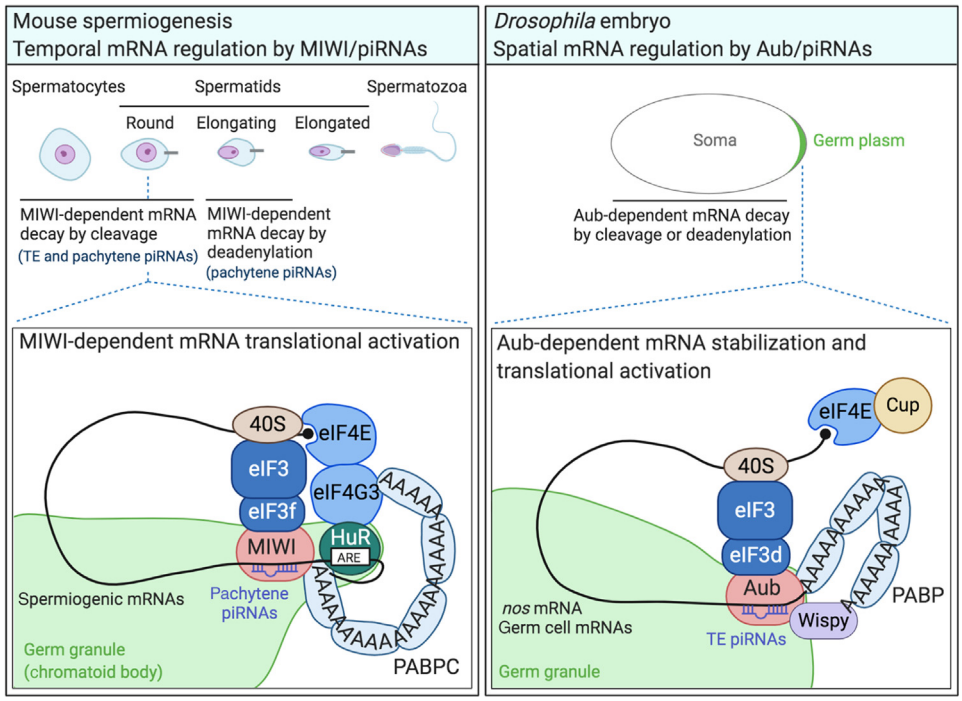

Figure 3. Translational Activation by Piwi-Interacting RNAs (piRNAs) and PIWI Proteins. Left panel: Temporal mRNA regulation by MIWI/piRNAs during mouse **spermiogenesis**. MIWI and piRNAs are key factors in mRNA regulation during spermiogenesis. They are involved in mRNA decay by MIWI-dependent cleavage in spermatocytes and round spermatids and by deadenylation through the recruitment of CAF1 in elongating spermatids. In addition, MIWI and HuR activate the translation of AU-rich element (ARE)-containing mRNAs in round spermatids. MIWI recruits the eIF3 translation initiation factor through direct interaction with eIF3f; it also directly associates with the PABPC that coats the mRNA poly (A) tail. HuR bound to the mRNA through ARE motifs recruits the eIF4G3 known to bridge the cap-binding protein eIF4E and PABPC, a process facilitating translation initiation. These protein interactions activate cap-dependent translation. The temporal regulation depends in part on stronger MIWI/eIF3f/HuR interactions in round spermatids and on stronger association between CAF1 and MIWI in elongating spermatids. Right panel: Spatial mRNA regulation by Aubergine (Aub)/piRNAs in the *Drosophila* embryo. *nos* maternal mRNA translation is repressed in the somatic part of the embryo and its targeting by piRNA/Aub contributes to its decay. By contrast, in the germ plasm (green), Aub promotes *nos* mRNA stabilization and translational activation. Aub recruits PABP by direct interaction and eIF3 at least via its association with the eIF3d subunit. Aub activates translation initiation, possibly by a cap-independent mechanism, bypassing the repression imposed by the eIF4E-binding protein Cup. The poly(A) polymerase Wispy that directly associates with Aub might also participate in translational activation through polyadenylation. In both the mouse and *Drosophila* systems, PIWI interactions with the translation machinery occur at the periphery of germ granules, suggesting localized translation at the edge of germ granules. TE, transposable element.

biogenesis and the presence of piRNA target sites in the *nos* 3′UTR. Polysome profiling revealed the role of Aub at the initiation step of translation [52].

These two studies identify a new function of PIWI proteins in promoting translation and strongly suggest evolutionary conservation of this function. Remarkably, several aspects of this PIWI mode of action show striking similarities in mouse and *Drosophila*. First, both MIWI and Aub activate translation at the level of initiation, by directly recruiting the translation initiation complex eIF3 [51,52] (Figure 3). Recent data have highlighted the regulatory functions of eIF3 in addition to its role in basal translation [54–56]. MIWI and Aub bind eIF3 through the eIF3f and eIF3d subunits, respectively. eIF3d plays an especially important role in the varied modes of eIF3-based regulation, involving both cap-dependent and -independent translation [57–59]. Second, both MIWI

and Aub directly associate with poly(A) binding protein (PABPC1 in the mouse; PABP in *Drosophila*), another actor in translation initiation [51,52,60]. Third, the localization of the PIWI protein interaction with eIF3 was similar in mouse and *Drosophila*. In both cases, immunostaining revealed eIF3 foci in close proximity to, rather that inside, PIWI-containing germ granules [51,52], suggesting that PIWI-dependent active translation occurs at the periphery of germ granules (Figure 3). These data reveal a high level of conservation of PIWI-dependent translational activation, adding a new fundamental PIWI mechanism of action in mRNA regulation, which might be conserved in other animal species.

piRNA-Independent Functions of PIWI Proteins

PIWI proteins are mostly specific to the germline; however, *PIWI* genes are aberrantly expressed in various types of human cancers and are part of the cancer-testis antigens that are testis-restricted genes induced in somatic cancers [61,62]. The mechanisms of action of PIWI proteins in cancers have remained puzzling because their expression is not linked to the presence of large amounts of piRNAs [63]. A recent study identified the function of PIWIL1 (the homolog of mouse MIWI) independent of piRNAs in pancreatic cancer metastasis [64]. Unloaded PIWIL1 was shown to interact with the anaphase-promoting complex/cyclosome (APC/C) that is involved in protein homeostasis through the ubiquitin-proteasome system. This interaction activates APC/C-dependent degradation of the cell adhesion-related protein Pinin, contributing to cancer cell migration and metastasis [64] (Figure 1D). A function of MIWI independent of piRNAs in protein regulation has also been described in mouse spermatogenesis. MIWI sequesters in the cytoplasm and inhibits the activity of the E3 ubiquitin ligase RNF8 known to play a role in histone ubiquitylation and degradation. The timely degradation of MIWI in late spermatids leads to RNF8's nuclear translocation and histone degradation and its replacement by protamines, a process essential for chromatin compaction and spermatid maturation [65] (Figure 1D). Although MIWI loading is not required for its interaction with RNF8, MIWI does associate with piRNAs in late spermatids and its loading is necessary for its degradation via APC/C [66]. This PIWI function is relevant to human male fertility, since mutations producing undegradable PIWIL1 were found in patients with azoospermia [65].

Finally, another piRNA-independent function of PIWIL1 was recently reported in gastric cancer. Although mRNA binding by PIWI proteins without piRNAs has been previously proposed in germ cells [13,18], this PIWI protein mode of action has not been formally demonstrated in germ cells and was not confirmed in subsequent studies [9,11,14]. piRNAs are not present in gastric cancer cells and PIWIL1 was shown to interact with mRNAs independent of piRNAs and repress them through the recruitment of nonsense-mediated mRNA decay components, promoting cancer cell proliferation, migration, and metastasis [67]. How PIWIL1 binds mRNAs without piRNAs remains to be analyzed.

These studies reveal emerging roles for PIWI proteins independent of piRNAs. More examples of such PIWI mechanisms of action remain to be discovered in the future, particularly in pathological contexts, where PIWI proteins are expressed independent of the whole cohort of genes required for piRNA biogenesis.

Concluding Remarks

Since their discovery as a germline-specific defense mechanism against TEs, piRNAs and PIWI proteins have been involved in a wide range of regulatory processes through the regulation of non-TE mRNAs. Because they were shaking up 'dogma', these PIWI/piRNA functions unrelated to TEs have been reluctantly accepted. However, it is unsurprising that, as an efficient system for mRNA decay, the piRNA pathway has been repurposed for cellular mRNA regulation. It is striking that the same developmental programs are regulated by piRNAs and PIWI proteins in very distant

Outstanding Questions

Given the low base pairing required for mRNA targeting by piRNAs, most mRNAs could be subject to piRNA-mediated silencing. How are specific mRNA regulation achieved and harmful off-target effects avoided? It is likely that varied mechanisms are at play in different biological contexts. Future studies should determine whether specificity involves cooperation with other regulatory mechanisms based on RNA-binding proteins or mRNA modifications, recurrent targeting of the same mRNA by multiple piRNAs, or additional mechanisms preventing piRNA-dependent silencing of specific mRNAs.

What is the contribution of piRNA-dependent mRNA regulation to the reproductive isolation of related species? To what extent does this function of piRNAs and PIWI proteins drive the adaptive evolution of the piRNA pathway and of mRNA sequences?

PIWI proteins interact with translation initiation factors and activate translation in very distant species (mouse and *Drosophila*). Is this PIWI function in translational activation conserved in other species?

PIWI proteins are present in RNA granules (nuage or germ granules in *Drosophila*, chromatoid bodies in the mouse) that are membraneless condensates assembling through liquid–liquid phase-separation principles. How are the biophysical properties of these granules linked to PIWI protein functions?

Can unloaded PIWI proteins bind the body of mRNAs and how is this achieved at the structural level, knowing that the structure of PIWI proteins allows interaction with both extremities of piRNAs or with one extremity of mRNAs?

PIWI proteins have different functions in protein homeostasis linked to the ubiquitin-proteasome system in mice and humans. Is this a widespread function of PIWI proteins? Is this function linked to pathological processes where piRNAs are not produced?

species. Biological functions of piRNA/PIWI-dependent mRNA regulation include germline development and specific processes linked to sexual reproduction such as sex determination and dosage compensation [28,29]. Massive maternal mRNA decay during the MZT in early embryos also depends on piRNAs and PIWI proteins in both *Drosophila* and *Aedes*, whose lineages separated 260 million years ago. Evolutionary studies are required to understand whether the regulation of these developmental programs by the piRNA pathway is conserved or was repeatedly co-opted for during evolution. Nonetheless, the presence of genic piRNAs across a wide range of species, including primitive bilaterian and non-bilaterian species, such as planarians and cnidarians, indicates that mRNA regulation by piRNAs and PIWI proteins is evolutionary ancient and conserved [8,11,16,17,20,23,68]. Many questions remain to be solved and a challenge for future studies is to understand how mRNA regulation by piRNAs and PIWI proteins is conserved although not at the sequence level (see Outstanding Questions). In contrast to miRNAs whose sequences are conserved through evolution, those of the piRNAs are not – even between closely related species. piRNAs are extremely diverse and their pool can evolve rapidly within a species (Box 1). In addition, the low level of base pairing required to target mRNAs indicates that most mRNAs might potentially be regulated by piRNAs. How is selectivity achieved? Cooperation with other regulatory pathways (e.g., RNA-binding proteins, mRNA modifications) are likely to contribute strongly. Consistent with this, piRNA-based regulation is often redundant with other regulatory mechanisms and involved in the fine-tuning of developmental processes [15]. Increasing the piRNA repertoire also provides novel opportunities for mRNA regulation that may be fixed (or discarded) by natural selection, leading to new piRNA biological functions. This capacity of piRNAs to evolve rapidly might also participate in speciation through reproductive isolation between related species mediated by both detrimental piRNA-dependent mRNA regulation and derepression of TEs in interspecies crosses.

Positive mechanisms of action of piRNAs and PIWI proteins in mRNA stabilization and translational activation have been discovered. Again, the parallels in the underlying mechanisms seem remarkable, with PIWI proteins recruiting the same translation initiation factors across very distant species. In addition, PIWI proteins act independently of piRNAs in cancer progression and metastasis. These studies reveal the high versatility of PIWI proteins in gene regulation and highlight the importance of these PIWI functions in developmental and pathological processes. Future analyses are likely to identify additional biological functions of piRNAs and PIWI proteins, particularly ones linked to germline development and sexual reproduction. They might also reveal new mechanisms of action of PIWI proteins and should allow better understanding of their connection with other regulatory mechanisms.

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