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REVIEW

piRNAs and PIWI proteins: regulators of gene expression in development and stem cells

Patricia Rojas-Ríos and Martine Simonelig*

ABSTRACT

PIWI proteins and Piwi-interacting RNAs (piRNAs) have established and conserved roles in repressing transposable elements (TEs) in the germline of animals. However, in several biological contexts, a large proportion of piRNAs are not related to TE sequences and, accordingly, functions for piRNAs and PIWI proteins that are independent of TE regulation have been identified. This aspect of piRNA biology is expanding rapidly. Indeed, recent reports have revealed the role of piRNAs in the regulation of endogenous gene expression programs in germ cells, as well as in somatic tissues, challenging dogma in the piRNA field. In this Review, we focus on recent data addressing the biological and developmental functions of piRNAs, highlighting their roles in embryonic patterning, germ cell specification, stem cell biology, neuronal activity and metabolism.

KEY WORDS: *Drosophila* embryonic patterning, Germ cells, Germline stem cells, Maternal mRNAs, Metabolism, Mouse spermatogenesis, mRNA localization, mRNA stability, piRNAs, PIWI proteins, Regeneration, Stem cells

Introduction

Piwi-interacting RNAs (piRNAs) are a class of small 21–32 nucleotide long non-coding RNAs present in animals ranging from sponges to humans. Like other small RNAs, piRNAs bind in their mature form to effector Argonaute proteins that form a specific clade and are collectively referred to as PIWI proteins. piRNAs were discovered and defined about a decade ago in mouse testes as small RNAs that interact with PIWI proteins (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006a). Although these mouse piRNAs were not initially linked to transposable elements (TEs), early reports in *Drosophila* revealed that a large proportion of piRNAs are generated from TE sequences and target TE mRNAs, thereby linking piRNA function to TE silencing in germlines (Brennecke et al., 2007; Gunawardane et al., 2007; Saito et al., 2006; Vagin et al., 2006). TEs are widespread in all genomes and can represent a substantial part of the genome. Owing to their mobility capacity and their presence in multiple copies in genomes, they play a major role in genome dynamics through mutational insertions and chromosome rearrangements. TEs are thus recognized as a driving force in genome evolution (Bourque, 2009; Britten, 2010; Feschotte, 2008). However, given their high proportion in the genomes, their transposition must be repressed to preserve genome integrity, and piRNAs are now known to play a key role in this repression in germlines.

piRNAs target TE mRNAs by a mechanism similar to that seen in other RNA-based silencing events: in brief, piRNAs bound to PIWI

proteins interact with their target mRNAs through base pairing, and the targeted mRNAs are then cleaved by the endonuclease activity of PIWI proteins (see Box 1 for details). Studies in *Drosophila* have shown that TE piRNAs are generated from large heterochromatic regions in the genome, which are composed of arrays of defective TEs, and in fact contain a repertoire of all the TEs present in the genome (Brennecke et al., 2007; Gunawardane et al., 2007). These arrays, which are termed piRNA clusters, are transcribed into long transcripts (either on one or on both strands) that are cleaved to produce piRNAs. These piRNAs then recognize TE mRNAs by complementarity, leading to the production of a second wave of piRNAs by PIWI-dependent cleavage, a process that consumes TE mRNAs, and is referred to as the ‘ping-pong’ cycle. Extensive progress in *Drosophila* and mouse models has contributed to deciphering the mechanisms of piRNA biogenesis and TE repression by piRNAs. Many new components have been identified and analyzed, following large RNAi screens in *Drosophila* (Czech et al., 2013; Handler et al., 2013). These data have been reviewed recently and we refer the reader to those reviews for this information (Ernst et al., 2017; Huang et al., 2017; Yamashiro and Siomi, 2018).

Although a large proportion of piRNAs are produced from TE sequences in some biological systems (e.g. 70% of piRNAs in *Drosophila* ovaries), this is not systematically the case. Genic piRNAs have been reported in gonads and embryos of various species (Mohn et al., 2015; Robine et al., 2009). These piRNAs are produced via the cleavage of a large number of mRNAs; they are produced in abundance from specific mRNAs (e.g. *traffic jam*, *brain tumor*) and tend to map to the 3'UTRs of these mRNAs (Robine et al., 2009; Saito et al., 2009). Furthermore, in specific contexts, piRNAs are generated from genomic clusters that are totally unrelated to TEs. This is the case in *C. elegans* germ cells and in adult mouse spermatogenesis. The existence of these non-TE-related piRNAs indicates additional functions for piRNAs that are independent of TE silencing. However, for several years, these piRNA functions have largely been overlooked. Nonetheless, evidence for piRNA functions in the regulation of gene expression have built up over the years, and several recent studies have substantially strengthened this aspect of piRNA biology, revealing novel functions for piRNAs in embryonic patterning, germ cell specification and stem cell biology, as well as in neurons and other somatic cells. In this Review, we discuss these functions of piRNAs that are independent of TE regulation, highlighting the role of piRNAs and PIWI proteins in post-transcriptional gene regulation, mRNA localization, stem cell biology and somatic tissues.

piRNAs in post-transcriptional gene regulation

Early insights from the *Drosophila* testes

An historical example of post-transcriptional gene regulation by piRNAs is the regulation of the *Stellate* (*Ste*) locus by *Suppressor of Stellate* [*Su(Ste)*] in *Drosophila* testes (Aravin et al., 2004, 2001). *Ste* is a repeated gene encoding a protein similar to the β -subunit of

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Box 1. Mechanisms of TE silencing by piRNAs

Transposable element (TE) mRNA cleavage by PIWI proteins upon targeting by piRNAs is one of the mechanisms leading to TE silencing. This mode of silencing generates piRNAs via a 'ping-pong' mechanism. PIWI proteins cleave targeted mRNAs 10 nucleotides upstream of the 5'-end of the guide piRNA, and generate the 5' end of a new piRNA in the opposite orientation. piRNA ping-pong pairs are characterized by a 10-nucleotide overlap at their 5' ends. In *Drosophila*, recent data have shown that ping-pong pairs can induce another mechanism that also consumes TE mRNAs. The first cleavage by the PIWI protein starts a series of downstream cleavages by Zucchini (Zuc), the endonuclease that is responsible for piRNA biogenesis from piRNA cluster transcripts (Han et al., 2015; Mohn et al., 2015). These Zuc-dependent cleavages occur without intervening nucleotides, generating piRNAs following one another along the mRNA. This process is referred to as phasing and also occurs on cellular mRNAs. In addition to these mechanisms of silencing at the post-transcriptional level, piRNAs repress TEs at the transcriptional level. piRNA loading into nuclear PIWI proteins (*Drosophila* Piwi; mouse Miwi2) induces their transport to the nucleus and binding to nascent RNAs through piRNA base pairing; this leads to TE transcriptional repression by DNA methylation or histone modification (Ernst et al., 2017; Yamashiro and Siomi, 2018). TE repression can also occur at the level of splicing. In *Drosophila*, the *P* element is regulated by splicing of its last intron, encoding either a transposition repressor in the absence of splicing or the *P* transposase upon splicing. A recent study has shown that piRNA-dependent repression of *P* does not occur through mRNA levels but through splicing of its last intron, potentially following chromatin remodeling (Teixeira et al., 2017).

casein kinase II. When expressed in testes, Ste protein forms elongated aggregates that affect spermatogenesis, leading to sterility. *Su(Ste)* is a repeated locus, related in sequence to *Ste*, that is located on the heterochromatic Y chromosome. *Su(Ste)* was identified genetically through its ability to repress *Ste* expression. This locus was recognized early on to produce small non-coding RNAs (Aravin et al., 2001). In fact, it is now known that *Su(Ste)* produces high levels of piRNAs complementary to *Ste* mRNA, which repress *Ste* expression through mRNA decay and translational repression (Kotelnikov et al., 2009). Although not related to TE silencing, piRNA-dependent regulation of the *Ste* gene has been widely used as a tool in early studies identifying new genes of the piRNA pathway, to confirm that these genes are indeed piRNA pathway components; mutations in such genes lead to accumulation of Ste protein aggregates in testes, as shown for *aubergine* (*aub*) and *argonaute 3* (*ago3*), which encode *Drosophila* PIWI proteins, and the piRNA pathway genes *zucchini* (*zuc*), *spindle-E* and *armitage* (Klattenhoff et al., 2007; Li et al., 2009; Pane et al., 2007; Tomari et al., 2004).

Another example of piRNA-mediated control of gene regulation involves *vasa* mRNA, which encodes the Vasa protein, a major determinant of the germline and component of the piRNA pathway. In *Drosophila* testes, *vasa* mRNA is regulated by piRNAs that are produced from non-TE repeated sequences on the X chromosome, called AT-chX. Notably, AT-chX piRNAs along with *Su(Ste)* piRNAs are the most abundant piRNAs in testes, representing another example where TE piRNAs do not predominate (Nagao et al., 2010; Nishida et al., 2007).

mRNA regulation by piRNAs during early embryogenesis in *Drosophila*

Independent studies of post-transcriptional gene regulation in the *Drosophila* embryo have demonstrated roles for piRNAs and the piRNA pathway in embryonic development, via the regulation of

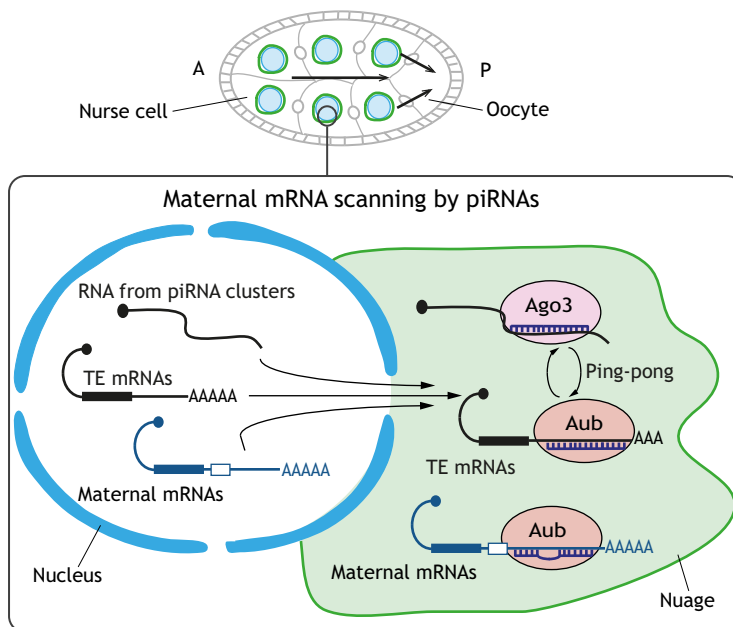
key developmental factors. Early embryogenesis, for about 2 h in *Drosophila*, occurs in the absence of zygotic transcription and depends on maternal mRNAs that are produced during oogenesis and transported into the future embryo (Fig. 1A,B). These maternal mRNAs are then massively degraded during the maternal-to-zygotic transition (MZT), when the control of development switches from the maternal to the zygotic genome. The mRNA *nanos* (*nos*), which encodes a key posterior determinant required for development of abdominal structures and the germline, was used as a paradigm to study mRNA decay during MZT. *nos* mRNA is present in the whole embryo but accumulates at the posterior pole, in the germ plasm – a specialized cytoplasm required for germ cell specification. *nos* is translationally repressed and degraded in the somatic part of the embryo, whereas it is stabilized and translated in the germ plasm (Fig. 1C). *nos* mRNA decay in the soma is known to depend on its deadenylation by the CCR4-NOT deadenylation complex, which is recruited by the RNA-binding protein Smaug (Zaessinger et al., 2006). However, a screen for candidates aimed at studying the role of RNA-silencing pathways in this regulation identified the piRNA pathway as a major mediator of *nos* mRNA decay during MZT (Rouget et al., 2010). This study showed that Aub and Ago3 are in complex with CCR4-NOT and Smaug in the early embryo, and are required for *nos* mRNA deadenylation. The interaction of Aub with *nos* mRNA is guided by piRNAs complementary to its 3'UTR. Importantly, piRNA-dependent regulation of *nos* mRNA contributes to its translational repression in the somatic part of the embryo and to embryonic patterning (Rouget et al., 2010). The major piRNAs that target *nos* mRNA are produced from two TEs, *roo* and *412*, highlighting the biological function of TEs in embryonic development through the regulation of essential developmental genes. This study established piRNA function in endogenous mRNA regulation for developmental processes, through incomplete base pairing in a mechanism similar to that used by miRNAs.

Using individual nucleotide UV crosslinking and immunoprecipitation (iCLIP) assays, Aub was subsequently found to directly bind several hundred maternal mRNAs in the early *Drosophila* embryo (Barckmann et al., 2015). The binding of Aub to cellular mRNAs requires its loading with piRNAs; a mutant form of Aub that is unable to load piRNAs does not bind mRNAs in iCLIP assays (Barckmann et al., 2015). Bioinformatic analyses identified a preferential interaction of Aub with mRNAs at the nucleotide in front of the 5'-most nucleotide of the guide piRNA. Interestingly, some mRNAs generate sense piRNAs upon targeting by a guide piRNA, with a ping-pong signature (10-nucleotide overlap between antisense and sense piRNAs). A similar phenomenon occurs in *Drosophila* ovaries in which a large number of cellular mRNAs produce piRNAs upon targeting by a highly complementary piRNA (Mohn et al., 2015). In this context, the guide piRNA first induces a PIWI-dependent cleavage, and this is followed by Zuc-dependent cleavages towards the downstream part of the mRNA by phasing, as for TE mRNAs (see Box 1). This reveals that the targeting of cellular mRNAs by piRNAs is widespread and suggests a general scanning of all maternal mRNAs by piRNAs (Barckmann et al., 2015). This scanning could occur in ovarian nurse cells, during the export of these mRNAs to the cytoplasm and passage through the nuage: an RNA granule structure that surrounds nurse cell nuclei and in which ping-pong takes place (Fig. 1A).

The functional importance of mRNA decay by Aub-dependent cleavage in embryonic development has also been confirmed for *dunce*, an mRNA that shows a strong ping-pong signature and encodes an enzyme involved in cAMP metabolism (Barckmann

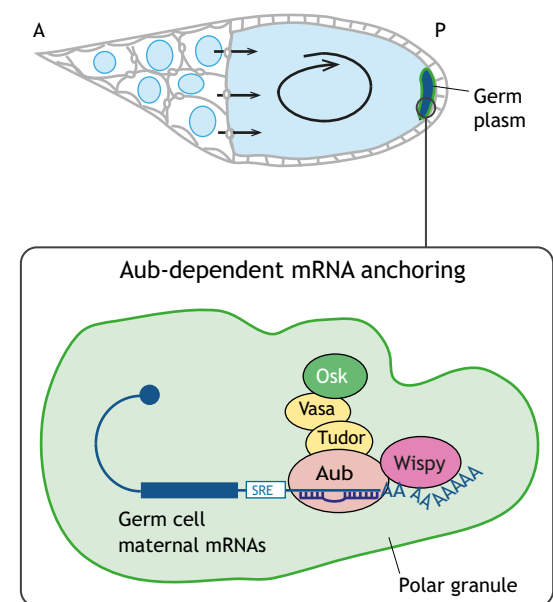
A Egg chamber, stage 8

mRNA transport from nurse cells to oocyte



B Egg chamber, stage 12

mRNA localization by diffusion and anchoring



C Embryo (0-2 h)

mRNA localization by decay and selective stabilization

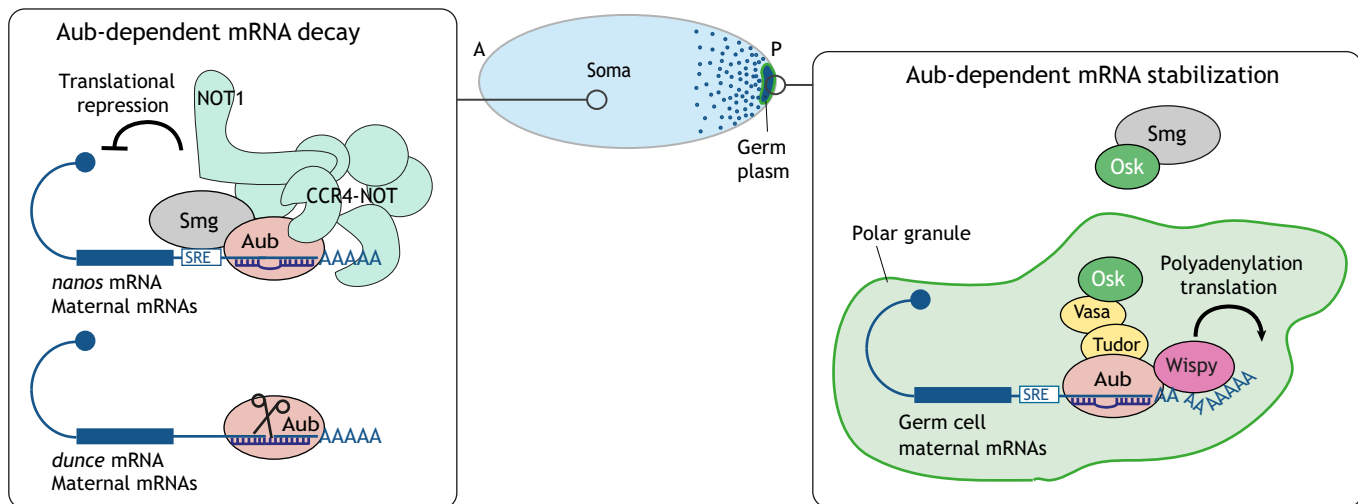


Fig. 1. Maternal mRNA regulation by Aub and piRNAs during *Drosophila* oogenesis and early embryogenesis. (A) mRNA scanning by piRNAs during oogenesis (stage 8). Egg chambers are composed of 15 nurse cells and one oocyte. The nuage (green), which is composed of ribonucleoprotein particles and in which the 'ping-pong' cycle takes place, assembles around nurse cell nuclei (blue). piRNAs loaded into Aub are proposed to perform transcriptome-wide scanning of transposable elements (TEs) and maternal mRNAs during their export to the cytoplasm through the nuage. Imperfect piRNA complementarity allows the recruitment of Aub to maternal mRNAs, which are then transported to the oocyte through ring canals (black arrows). (B) Maternal mRNA localization by Aub and piRNAs during oogenesis (stage 12). Nurse cell cytoplasm is poured into the oocyte through ring canals (black horizontal arrows). This contributes to a rotating flow in the cytoplasm of the oocyte (black circular arrow), allowing diffusion of maternal mRNAs and their localization to the germ plasm by anchoring. Polar granules that are ribonucleoprotein particles related to nuage assemble in the germ plasm and contain Aub, its interactor Tudor, as well as Vasa and Oskar. Aub binds maternal mRNAs through piRNA incomplete base pairing, which anchors them to the germ plasm. Wispy poly(A) polymerase colocalizes with Aub in the germ plasm. This mechanism of localization is inefficient and a large proportion of mRNA-protein complexes remains in the cytoplasm of the oocyte. (C) Dual role of piRNAs and Aub in maternal mRNA decay in the soma and stabilization in the germ plasm. Smaug (Smg) is expressed in early embryos and binds maternal mRNAs through Smg recognition elements (SRE). Aub is also bound to maternal mRNAs through piRNA base pairing (A,B). Smg and Aub recruit the CCR4-NOT complex, which induces mRNA deadenylation and decay. CCR4-NOT is also involved in translational repression independently of deadenylation (Götze et al., 2017). In addition, Aub can induce mRNA decay by direct cleavage. In the germ plasm, Smg is sequestered by Oskar, preventing its binding to maternal mRNAs. Aub directly interacts with the Wispy poly(A) polymerase, inducing mRNA poly(A) tail elongation and stabilization. Polyadenylation also likely contributes to the translation of mRNAs localized to the germ plasm. Nos protein is indicated as blue dots. A, anterior; P, posterior.

et al., 2015). The binding of Aub to maternal mRNAs in early embryos can lead to mRNA decay by two mechanisms: either deadenylation via the recruitment of the CCR4-NOT complex or cleavage by the endonuclease activity of Aub. These two outcomes might depend on the level of base pairing with guide piRNAs, in particular at nucleotides 10–11, between which cleavage occurs. Overall, these results identify piRNAs and Aub as key factors of maternal mRNA decay during the MZT. Strikingly, maternal mRNAs subject to piRNA/Aub-dependent decay accumulate in the germ plasm and are required for germ cell development, highlighting a key role for piRNAs in the specification and development of the germline through the co-regulation of mRNAs involved in this developmental program (Barckmann et al., 2015) (Fig. 1C).

mRNA regulation by piRNAs in mouse spermatogenesis

The regulation of non-TE mRNAs by piRNAs also takes place during mouse spermatogenesis. piRNAs that are produced from TE sequences and are called pre-pachytene piRNAs are present in mouse germ cells in the male gonad before birth and during the neonatal period. These piRNAs are loaded into two mouse PIWI proteins: Miwi2 and Mili (Fig. 2). *Miwi2* and *Mili* mutants stop spermatogenesis, with spermatocytes arresting at the leptotene and zygotene/early pachytene stages, respectively, and exhibiting TE upregulation (Carmell et al., 2007; De Fazio et al., 2011; Shoji et al., 2009). In contrast, pachytene piRNAs, which are produced in spermatocytes after birth, are largely unrelated to TEs and ~75% are generated from non-repetitive sequences and endogenous mRNAs (Aravin et al., 2006; Beyret et al., 2012; Girard et al., 2006; Grivna et al., 2006a; Reuter et al., 2011; Robine et al., 2009). The vast majority of pachytene piRNAs are loaded into a third PIWI protein, Miwi, the expression of which is concomitant with their appearance (Li et al., 2013) (Fig. 2). Spermatogenesis arrest in *Miwi* mutants occurs at a later stage, in round spermatids (Deng and Lin, 2002; Reuter et al., 2011). Although deregulation of LINE1 TEs is observed in *Miwi* mutants (Reuter et al., 2011), the lack of a relationship between most pachytene piRNAs and TE sequences prompted analyses to decipher their function (Gou et al., 2014), revealing key insights into the roles of piRNAs during spermatogenesis.

In late spermatocytes and round spermatids, the reorganization of chromatin – from a closed structure to a more open structure – correlates with the widespread expression of long noncoding RNAs (lncRNAs). A recent study has shown that pachytene piRNAs target lncRNAs, as well as protein-coding mRNAs, and induce their repression through the slicer activity of Miwi (Watanabe et al., 2015). This regulation involves a small fraction of pachytene piRNAs produced from defective TEs; these piRNAs target the same ancient TEs that are inserted into lncRNAs and the 3'UTRs of mRNAs (Fig. 2A). Other mRNAs are regulated through base-pairing with piRNAs generated from their cognate pseudogenes. This piRNA-dependent repression affects mRNAs expressed from early spermatocytes that tend to have longer 3'UTRs containing defective TE insertions (Watanabe et al., 2015).

During the later stages of spermatogenesis, referred to as spermiogenesis, a complex differentiation program allows the differentiation of round spermatids into mature sperm. This process involves widespread changes in the transcriptome, including massive mRNA decay (Zuo et al., 2016). CLIP assays have shown that Miwi in round spermatids binds to a large proportion of endogenous mRNAs (Vourekas et al., 2012; Zhang et al., 2015). Moreover, analyses of base pairing between pachytene piRNAs and Miwi-bound mRNAs identified ping-pong signals at mRNA cleavage sites, thus unambiguously demonstrating endogenous

mRNA targeting by piRNAs. Consistent with this, mRNA decay by Miwi was shown to involve its slicing activity (Zhang et al., 2015) (Fig. 2B). Testis lentiviral transduction with piRNA-resistant constructs indicated that piRNA-dependent mRNA decay is required for progression to mature sperm (Zhang et al., 2015). The binding of Miwi to mRNAs independently of piRNAs was also proposed to occur in round spermatids (Vourekas et al., 2012). However, as Miwi mRNA-binding is piRNA-dependent at all stages of Miwi expression, whether two modes of Miwi binding indeed co-exist in round spermatids awaits further studies. In addition, although piRNA-independent Miwi binding was reported to result in the stabilization of mRNAs in a translation-repressed state (Vourekas et al., 2012), the analysis of mice mutant for *maelstrom*, which encodes a factor that is required for piRNA biogenesis, suggested a role – potentially indirect – for piRNAs in the translational activation of spermiogenic mRNAs at the same stage (Castaneda et al., 2014). Whether and how Miwi and piRNAs might be involved in these different regulatory mechanisms represent challenging questions and will undoubtedly be clarified in the near future.

The analysis of mRNA regulation by piRNAs during the next stage of spermatogenesis, in elongating spermatids, identified massive endogenous mRNA decay through piRNA targeting and binding of Miwi. In this context, Miwi acts by directly recruiting the CAF1 deadenylase, a component of the CCR4-NOT complex, inducing mRNA deadenylation and decay (Gou et al., 2014) (Fig. 2C). Therefore, as in the *Drosophila* embryo, mRNA targeting by piRNAs in mouse male germ cells leads to decay either by Miwi-dependent cleavage or by deadenylation. Notably, the base pairing requirement to support cleavage is higher (with a strong match at nucleotides 1–16; fewer than three mismatches at nucleotides 2–20) than that needed for deadenylation (which requires seed match at nucleotides 2–7, followed by loose complementarity) (Gou et al., 2014; Zhang et al., 2015). In an elegant gain-of-function approach, ectopic pachytene piRNAs were generated through insertion of a human piRNA cluster in the mouse genome. Strikingly, this led to male sterility because of the reduced levels of mRNAs required for spermatogenesis (Goh et al., 2015). The role of a specific mRNA, *Dpy19L2*, which is required for formation of the sperm acrosome (a sperm-specific organelle that is required for fertilization), was confirmed by the rescue of male fertility using a human *Dpy19L2* transgene resistant to piRNAs. In this context, mRNA decay through Miwi-dependent slicing and ping-pong was also reported, as well as the requirement for high complementarity between piRNAs and mRNAs (i.e. a perfect match at nucleotides 2–11; fewer than five mismatches at nucleotides 12–21). This study further confirmed endogenous mRNA destabilization by Miwi and pachytene piRNAs.

Together, these reports reveal that a huge repertoire of pachytene piRNAs play a key role in the general mRNA decay occurring during spermiogenesis. The temporal regulation of this process relies on the proteasomal degradation of Miwi in late spermatids, which also leads to piRNA destabilization (Zhao et al., 2013). Miwi has an additional function in the timely histone-to-protamine transition in late spermatids. It binds to the E3 ubiquitin ligase RNF8, which is involved in histone ubiquitylation and degradation, and sequesters it in the cytoplasm; the degradation of Miwi in late spermatids allows RNF8 nuclear translocation, histone degradation and histone replacement by protamines (Gou et al., 2017). These findings suggest that Miwi and its *Drosophila* homolog Aub might be unique among PIWI proteins as they both bind endogenous mRNAs. The parallel between them extends to their function in massive mRNA decay during specific developmental transitions, and their mechanisms of action involving both cleavage and deadenylation.

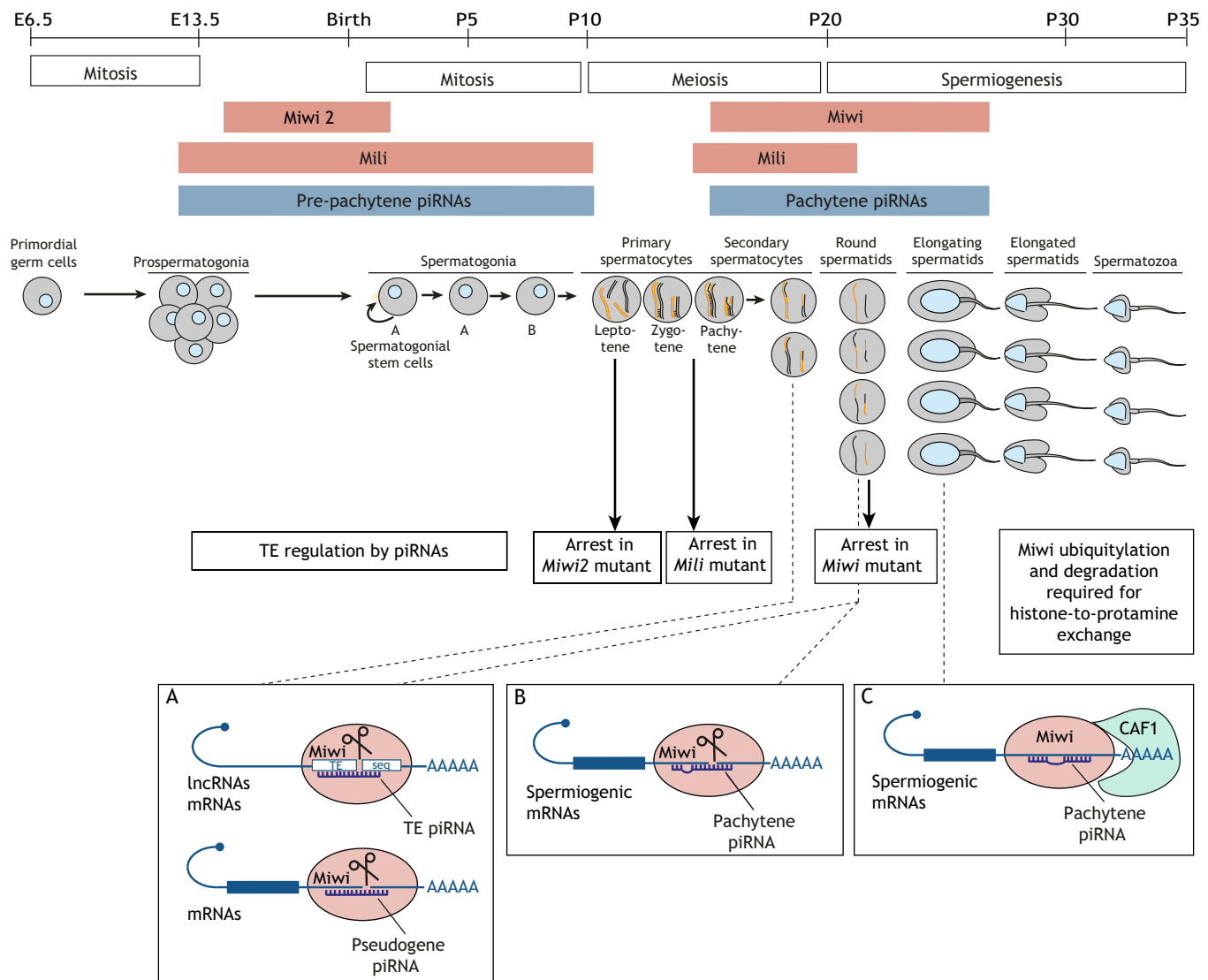


Fig. 2. mRNA regulation by piRNAs and Miwi during mouse spermatogenesis. A schematic of spermatogenesis is shown in the top part of the figure. The scale is in days (E, embryonic; P, postnatal). Expression of the three PIWI proteins (pink) during spermatogenesis is very specific and dynamic. Mili expression starts at E12.5 in prospermatogonia and extends during the neonatal period up to the beginning of meiosis. Miwi2 is also expressed in embryonic stages from E14.5 up to birth. Mili and Miwi2 bind to and are involved in the biogenesis of pre-pachytene piRNAs that are produced from transposable element (TE) sequences and regulate TEs. Expression of Mili is reactivated later during meiosis, at the zygotene-to-pachytene transition, while Miwi expression starts at the pachytene stage together with expression and biogenesis of pachytene piRNAs. The vast majority of pachytene piRNAs are loaded into Miwi; these piRNAs are mostly produced from sequences unrelated to TEs. Miwi is degraded during spermiogenesis in elongated spermatids, and this plays a role in histone ubiquitylation and degradation that is required for histone-to-protamine exchange and chromatin compaction in sperm. (A) The small proportion of pachytene piRNAs produced from TE sequences and pseudogenes target TEs inserted in lncRNAs or mRNAs, and cognate mRNAs, respectively, leading to Miwi-dependent mRNA cleavage and decay in late spermatocytes and round spermatids. (B,C) The bulk of pachytene piRNAs target spermiogenic mRNAs in round and elongating spermatids, leading to either Miwi-dependent cleavage (B) or recruitment of the CAF1 deadenylase and mRNA decay (C).

The conservation of endogenous mRNA regulation by piRNAs

The presence of a large repertoire of TE-independent piRNAs in adult testes is conserved in many species, including hen, rat, pig, marmoset and human (Gebert et al., 2015; Hirano et al., 2014; Lau et al., 2006; Li et al., 2013; Williams et al., 2015). In addition, PIWI proteins bind a large proportion of endogenous mRNAs in oocytes from two *Xenopus* species (Toombs et al., 2017). This set of data indicates a conserved biological function of piRNAs in post-transcriptional regulation of developmental programs. In the *C. elegans* germline, piRNAs broadly engage in germline mRNA targeting and regulation, through the generation of secondary small RNAs (Box 2). This allows the identification of germline-expressed and non-expressed mRNAs across generations. Furthermore, recent

evolutionary analyses of the piRNA pathway have revealed that the function of piRNAs in endogenous mRNA regulation is ancestral (Lewis et al., 2018; Praher et al., 2017).

Another compelling example is the mandatory role of female-specific piRNAs for the female fate in *Bombyx mori* (Kiuchi et al., 2014). These piRNAs are produced from repeated sequences present on the female sex chromosome and repress the *Masculinizer* (*Masc*) gene localized on the male sex chromosome, which is reminiscent of the regulation of *Ste* by *Su(Ste)* in *Drosophila* testes. *Masc* is involved in the production of the male-specific variant of *doublesex* and thus in male differentiation, as well as in dosage compensation in males. The female-specific piRNAs target and repress *Masc* mRNA through cleavage by the PIWI protein Siwi,

Box 2. Transcriptome-wide mRNA regulation by piRNAs in the *C. elegans* germline

In *C. elegans*, piRNAs that are 21 nucleotides long and called 21U-RNAs are not derived from transposable element sequences. They are loaded into the PIWI protein PRG-1 and target germline mRNAs through incomplete base pairing (Bagijn et al., 2012; Lee et al., 2012; Weick et al., 2014). In contrast to *Drosophila* or mammals, mRNA targeting by piRNAs/PRG-1 in *C. elegans* induces the production of secondary antisense small RNAs (called 22G-RNAs) by RNA-dependent RNA polymerases. This allows mRNAs that are regulated by piRNAs to unambiguously be identified. In turn, 22G-RNAs are loaded into Argonaute proteins of either the WAGO group, which induce transcriptional or post-transcriptional mRNA target repression, or CSR-1, which prevents silencing through increased transcription (Ashe et al., 2012; Cecere et al., 2014; Phillips et al., 2015; Seth et al., 2013; Shirayama et al., 2012; Wedeles et al., 2013). 22G-RNAs are produced from most germline-expressed genes, indicating transcriptome-wide mRNA regulation by piRNAs. This process is involved in the recognition of expressed (self) transcripts through transgenerational CSR-1 memory, versus silenced transcripts/foreign sequences (non-self), in the germline across generations. Recent studies analyzing the rules of piRNA base pairing with mRNAs confirmed the requirement in *C. elegans* of strong complementarity in the seed region (nucleotides 2-8) followed by an additional match at nucleotides 14-19 (Shen et al., 2018; Zhang et al., 2018).

thereby inducing the production of piRNAs from *Masc* mRNA and piRNA amplification by ping-pong in female embryos (Kiuchi et al., 2014). Mutations that decrease the levels of these female-specific piRNAs lead to female masculinization, highlighting the key role of this piRNA-dependent regulation in sex determination. Strikingly, a recent study described similar piRNA-dependent regulation of sex determination and dosage compensation in two nematode species (Tang et al., 2018), leading to the thrilling hypothesis that the piRNA-mediated regulation of very specific developmental processes might be evolutionarily conserved.

piRNAs in mRNA localization

In the early *Drosophila* embryo, the regulation of germ cell mRNAs by piRNAs is spatially controlled, and this tight spatial regulation is required for both embryonic patterning and germ cell specification. At this stage, zygotic nuclei divide in a single cell, forming a syncytium, but the cytoplasm is already regionalized: germ plasm is found at the posterior pole and is required for germline specification, whereas the rest of the embryo will form the soma. Germ plasm is composed of specific ribonucleoprotein granules (called polar granules in *Drosophila* and chromatoid bodies in other species), which are conserved structures found in germ cells of many species and contain mRNAs essential for germ cell specification and development. In *Drosophila*, germ plasm starts to assemble at the posterior pole of the oocyte during oogenesis, beginning with the localization of *oskar* mRNA, which encodes the primary germ plasm determinant (Ephrussi and Lehmann, 1992). The bulk of germ cell mRNA localization to the germ plasm then occurs during late oogenesis through an mRNA diffusion and trapping mechanism (Fig. 1B). However, this mechanism is inefficient and mRNA localization in the embryo is refined through the selective decay of unlocalized mRNAs present in the soma (Martin and Ephrussi, 2009) (Fig. 1C). Strikingly, Aub is involved in germ cell mRNA decay in the soma but is also a core component of polar granules in the germ plasm (Little et al., 2015; Thomson et al., 2008; Trcek et al., 2015; Zheng et al., 2016). This raises the intriguing issue of

whether Aub might be involved in two opposite functions depending on its localization: mRNA decay in the somatic part of the embryo and mRNA localization in the germ plasm (Barckmann et al., 2015). In line with this, Aub-CLIP assays identified widespread binding of Aub to germ cell mRNAs (Barckmann et al., 2015; Vourekas et al., 2016), and this finding, together with the observed accumulation of Aub in polar granules, led to the proposed role of Aub in mRNA trapping to the germ plasm during late oogenesis (Vourekas et al., 2016). The analysis of piRNA base pairing with mRNAs revealed high interactions at nucleotides 2-6 and 16-24, reminiscent of the rules described for mouse piRNAs (Vourekas et al., 2016). Because this loose base pairing could potentially allow transcriptome-wide Aub binding, these interactions were reported to occur randomly, in a 'sequence non-specific' manner, with the specificity to germ cell mRNAs relying on the longer size of these mRNAs providing more target sites for piRNAs (Vourekas et al., 2016). The tendency to be longer is a conserved feature among germ cell mRNAs in drosophilids, suggesting it could participate in selective Aub binding. However, detailed studies of the localization of specific germ cell mRNAs, e.g. *nos*, have shown that it is a complex process involving several redundant factors that most likely cooperate with Aub for specific binding (Becalska et al., 2011; Sinsimer et al., 2011). In addition, the analysis of *nos* mRNA localization showed that the deletion of two piRNA target sites in the *nos* 3'UTR affects localization to the germ plasm to some extent, indicating the contribution of specific piRNAs (Dufourt et al., 2017).

In *aub* mutant embryos, germ plasm assembly at the posterior is prevented due to developmental defects occurring during oogenesis (Klattenhoff et al., 2007). The role of Aub in mRNA localization to the germ plasm has thus been demonstrated using *aub* mutant embryos in which an ectopic germ plasm assembles at the anterior pole: in this context, germ cell mRNAs do not localize to the germ plasm in the absence of Aub (Dufourt et al., 2017). The molecular mechanisms underlying the switch in Aub function between soma and germ plasm have also been addressed. Whereas Aub participates in mRNA deadenylation and decay in the soma through recruitment of the CCR4-NOT deadenylation complex, it directly interacts with Wispy, the germline-specific poly(A) polymerase (Benoit et al., 2008), leading to germ cell mRNA poly(A) tail elongation and stabilization in the germ plasm (Dufourt et al., 2017) (Fig. 1C). The molecular events allowing this switch in Aub complex activity between the two compartments are not fully understood. Polar granules contain many specific proteins, including Oskar, Vasa and the direct Aub interactor Tudor, which are likely to play a key role in Aub complex remodeling and may regulate Wispy activity. In addition, the CCR4 deadenylase is depleted in the germ plasm (Dufourt et al., 2017).

Overall, these data reveal an unexpected role for piRNAs in mRNA stabilization and raise the issue of how this could be achieved. Loose piRNA base pairing resulting in a bulge around nucleotides 10-11, at which slicing takes place, would not allow Aub-dependent mRNA cleavage; Aub might thus recruit deadenylation or polyadenylation complexes depending on the context, as RNA-binding proteins do. However, extensive piRNA base pairing to mRNAs has also been reported and shown to result in mRNA cleavage (Barckmann et al., 2015; Mohn et al., 2015). In that case, mRNA stabilization could rely on either an inefficient nucleolytic activity of Aub, or additional factors that prevent this activity, already present in ovaries. Consistent with this, it was recently shown that mRNAs from specific TEs accumulate and localize to the germ plasm in *p53* mutant ovaries, even though the piRNA pathway is functional (Tiwari et al., 2017).

Interestingly, PIWI-dependent mRNA localization to chromatoid bodies is conserved in planaria, an organism showing extreme regenerative capacity through a population of adult stem cells called neoblasts (Elliott and Sánchez Alvarado, 2013). PIWI homologs are markers of neoblasts and are required for their maintenance and function (Palakodeti et al., 2008; Reddien et al., 2005). They are also involved in the localization of *histone H4* mRNA to chromatoid bodies (Rouhana et al., 2014). In line with this, the knockdown of planarian PIWI proteins results in both increased levels of histone H4 mRNA and delocalization from chromatoid bodies, suggesting a potential dual role for planarian PIWIs in mRNA stabilization/destabilization, as appear to be the case for Aub.

piRNAs and PIWI proteins in stem cell biology

The *piwi* (*P*-element-induced wimpy testis) gene was initially identified in a *Drosophila* *P*-element enhancer trap screen and was shown to be involved in the asymmetric division of germline stem cells (GSCs) (Lin and Spradling, 1997). Accordingly, *piwi* mutants show rudimentary gonads and sterility, owing to strong defects in gametogenesis and maintenance of GSCs in both females and males (Gonzalez et al., 2015; Lin and Spradling, 1997). Piwi represents the founding member of the conserved PIWI clade of Argonaute proteins. The conservation extends to the functions of PIWI proteins in GSC proliferation and maintenance, from *C. elegans* and *Drosophila* to the mouse (Cox et al., 1998; Unhavaithaya et al., 2009).

In *Drosophila* females, GSCs are localized at the anterior-most region of the ovary and self-renew throughout adult life to give rise to all germ cells. They are in contact with niche somatic cells termed cap cells and escort cells, which provide signaling cues to GSCs for their self-renewal and differentiation (Fig. 3A). GSCs divide asymmetrically to produce a new stem cell that remains in contact with cap cells, and another cell that differentiates into a cystoblast upon losing this contact. This cell fate transition is regulated by the BMP signaling molecule Dpp, which is secreted from cap cells and leads in GSCs to transcriptional repression of *bag of marbles* (*bam*), which encodes the master GSC differentiation factor. Dpp diffusion is highly regulated, allowing Bam expression in cystoblasts and activation of the differentiation program. Strikingly, Piwi is expressed and has functions in both somatic niche cells and germ cells. In addition, these functions impact both GSC self-renewal and differentiation. Piwi is highly expressed in GSCs and downregulated in cystoblasts and dividing cysts, in a reciprocal expression pattern to Bam (Cox et al., 2000; Dufourt et al., 2014; Szakmary et al., 2005). Piwi is intrinsically required in germ cells before adulthood for primordial germ cell maintenance or GSC establishment in the gonad, and in adult GSCs for their division, maintenance and differentiation (Cox et al., 2000; Ma et al., 2014). Early studies addressing the role of Piwi in somatic cells showed its requirement in the niche to repress Bam expression in GSCs – through BMP or another signaling pathway – and thus promote GSC self-renewal (Cox et al., 2000; Szakmary et al., 2005). Then, Piwi was shown to act in escort cells for GSC differentiation, in part by reducing BMP signaling, allowing Bam expression in cystoblasts (Jin et al., 2013; Ma et al., 2014). Piwi is also required in somatic cells in the larval gonad for their intermingling with germ cells (Jin et al., 2013; Saito et al., 2009).

More recent studies have addressed the molecular mechanisms underlying Piwi functions in GSCs and somatic niche cells. One mechanism involves Piwi interaction with Polycomb-group proteins of PRC2, a complex involved in epigenetic repression through histone methylation. Piwi interaction with PRC2 was proposed to sequester the complex in the nucleoplasm, leading to reduced PRC2

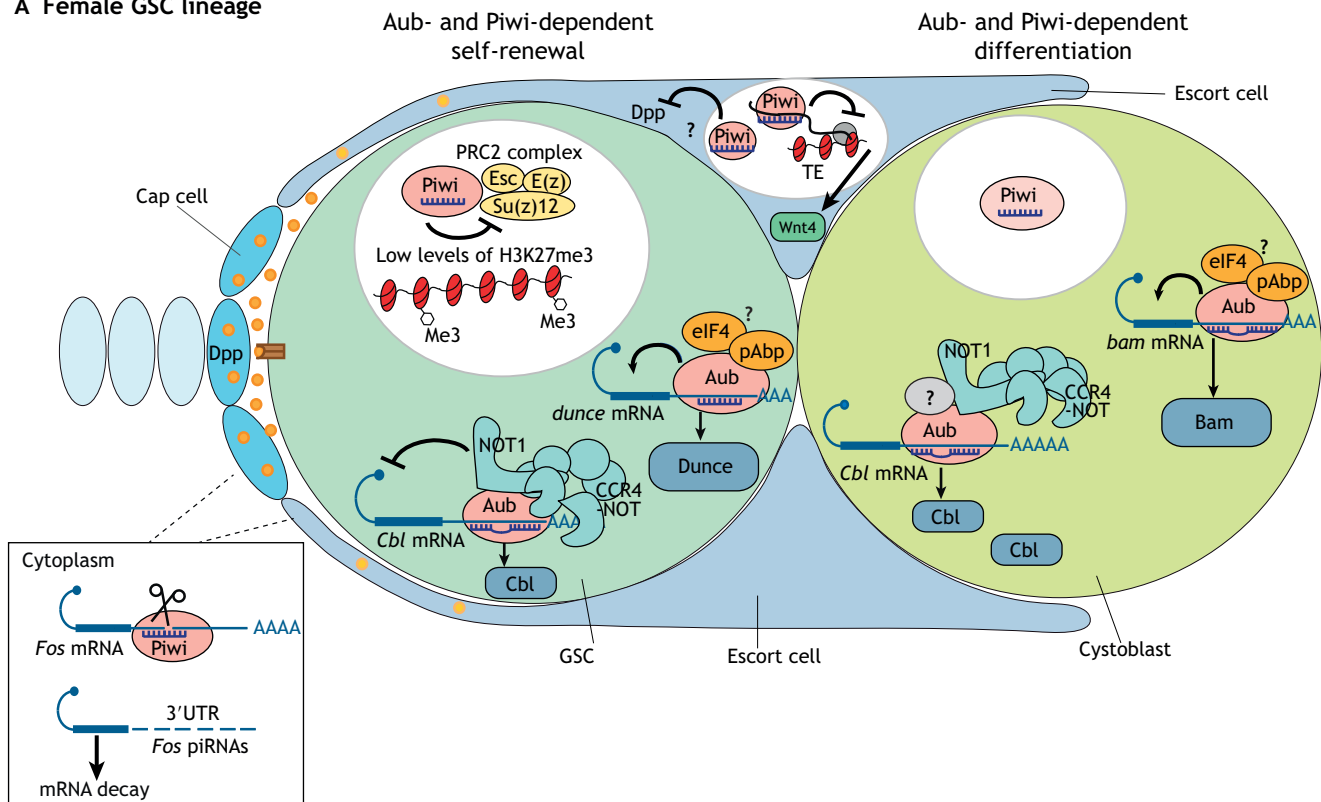
binding to chromatin. Therefore, Piwi plays an indirect role in gene regulation by limiting PRC2-dependent transcriptional repression (Peng et al., 2016) (Fig. 3A). In somatic niche cells, *Fos* repression by Piwi at the mRNA level was shown to contribute to Piwi function in GSC self-renewal and differentiation (Klein et al., 2016). Piwi binds the *Fos* 3'UTR, inducing its cleavage into piRNAs and the decay of *Fos* mRNA (Fig. 3A). Reducing *Fos* gene dose partially rescues the self-renewal and differentiation defects of *piwi* mutants, revealing the functional importance of *Fos* regulation by Piwi for GSC homeostasis (Klein et al., 2016). These results imply a role for Piwi in the cytoplasm, and might be consistent with another study suggesting that nuclear localization of Piwi in niche cells is not required for GSC self-renewal (Klenov et al., 2011).

The analysis of Piwi function in *Drosophila* testes has identified a role for Piwi not only in GSCs for their self-renewal (Gonzalez et al., 2015), but also in somatic stem cells called cyst stem cells, which are required to support germline cells. Cyst stem cells give rise to somatic cyst cells, the functional equivalent of ovarian escort cells. Specifically, Piwi has been shown to be necessary for cyst stem cell self-renewal (Gonzalez et al., 2015). Molecular analyses have shown that Piwi is loaded predominantly with genic piRNAs in the *Drosophila* testis, among which are antisense piRNAs to the first intron of *Fasciclin 3* (*Fas3*), which encodes an adhesion molecule important for gonadal development (Fig. 3B). Piwi represses *Fas3* in somatic cyst cells, through its nuclear function and possibly *Fas3*-targeting piRNAs (Gonzalez et al., 2015). Similarly, Piwi has been shown to repress *Fas3* in somatic cells of the female larval gonad, potentially via incomplete base pairing in the first intron with genic piRNAs from *traffic jam* (Saito et al., 2009).

These studies establish that the functions of Piwi in GSC biology largely depend on the regulation of gene expression at the transcriptional and post-transcriptional levels. However, a key issue is whether TE regulation by the piRNA pathway also contributes to GSC homeostasis. It is known that TE derepression either in germ cells by *PM*-hybrid dysgenesis – a syndrome resulting from *P*-element transposition – or in somatic cells using a mutant of *flamenco*, the major somatic piRNA cluster, leads to GSC tumors due to defects in differentiation (Rangan et al., 2011). More recently, the role of TE regulation by piRNAs has been addressed in somatic niche cells using the knockdown of *eggless* (which encodes a histone methyltransferase required for piRNA cluster transcription), *piwi* knockdown or *flamenco* mutants. This study showed that all three conditions lead to downregulation of the Wnt ligand Wnt4 in escort cells, resulting in GSC differentiation defects (Upadhyay et al., 2016), supporting the notion that TE repression participates in Wnt4 expression and signaling within escort cells for GSC differentiation (Fig. 3A). In addition, TE expression and DNA damage are increased in *piwi* knockdown escort cells, which might lead to defects in these cells and, in turn, to defective cystoblast differentiation (Ma et al., 2014). In conclusion, the regulation of both TEs and endogenous mRNAs by Piwi appear to mediate its function in GSC biology.

Two recent studies have analyzed the role of Aub in ovarian GSC biology (Ma et al., 2017; Rojas-Ríos et al., 2017), revealing that *aub* mutants show defects in both GSC self-renewal and differentiation. Aub expression is restricted to germ cells, and genetic studies have confirmed the intrinsic role of Aub in GSCs (Ma et al., 2017; Rojas-Ríos et al., 2017). It is known that the Chk2-dependent DNA damage checkpoint is activated in several piRNA pathway mutants, leading to various developmental defects during oogenesis (Klattenhoff et al., 2007; Pane et al., 2007); these defects are partially rescued in *Chk2* mutants. DNA damage in piRNA pathway

A Female GSC lineage



B Male GSC lineage

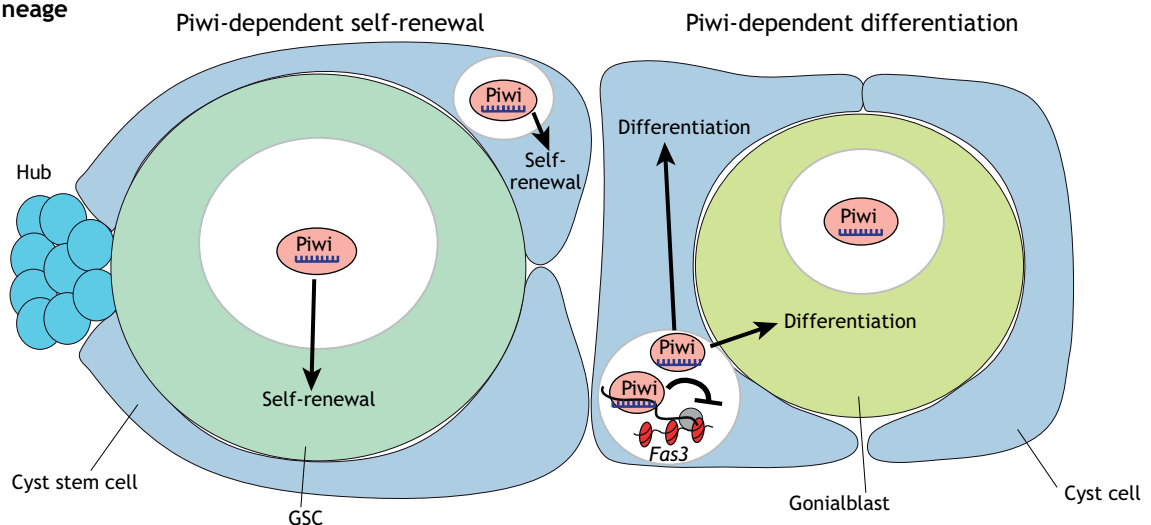


Fig. 3. Aub and Piwi functions in the biology of *Drosophila* female and male GSCs. (A) In the ovary, Piwi is expressed in both somatic niche cells and germ cells, whereas Aub expression is restricted to germ cells. Piwi expression is reduced (lighter color) in cystoblasts. In GSCs, Piwi is autonomously required for GSC self-renewal through indirect regulation of gene expression. Piwi sequesters the PRC2 complex, reducing its suppressive activity through low deposition of H3K27me3 chromatin marks. Piwi can also directly bind DNA. In escort cells, Piwi represses *Dpp* through an unknown mechanism (indicated by a question mark). In addition, Piwi represses *Fos* mRNA in the cytoplasm of cap and escort cells by binding its 3'UTR and inducing its cleavage into piRNAs, resulting in *Fos* mRNA decay. Piwi also regulates *Wnt4* expression in escort cells through repression of transposable elements (TEs) at the transcriptional level. This could occur through piRNA base pairing with TE nascent RNAs; RNA Pol II is represented as a gray circle. *Wnt4* autocrine signaling in escort cells is involved in encapsulation of cystoblasts, which contributes to their differentiation. Aub is autonomously required in GSCs for their self-renewal and differentiation. Aub binds *Cbl* mRNA through incomplete base pairing with piRNAs and recruits the CCR4-NOT deadenylation complex. This leads to *Cbl* mRNA translational repression potentially directly via CCR4-NOT. In cystoblasts, Aub-dependent repression of *Cbl* is reduced or abolished, leading to increased levels of this differentiation factor. This likely involves the action of unknown factors (question mark). Aub has a positive role in the regulation of *dunce* and *bam* mRNAs in GSCs and cystoblasts, respectively. This regulation is likely to involve piRNA base pairing, and might occur through the recruitment of translation initiation factors and pAbp. (B) In the testes, Piwi is required in GSC and somatic cyst stem cells for their self-renewal. In somatic cyst cells, Piwi has a role in the nucleus for their differentiation, as well as for germ cell differentiation. Piwi represses *Fas3* mRNA in these cells, possibly through piRNA base-pairing within the first intron. This repression occurs at the transcriptional level, likely via piRNA targeting of *Fas3* nascent transcript; RNA Pol II is represented as a gray circle.

mutants is thought to result from TE mobilization, and indeed the defects in GSC differentiation observed in *PM*-hybrid dysgenesis are partly rescued by a mutation in *Chk2* (Rangan et al., 2011). *aub* mutant GSC phenotypes are also partly rescued by the *Chk2* mutant, indicating the contribution of DNA damage to these defects, perhaps following TE derepression (Ma et al., 2017; Rojas-Ríos et al., 2017). However, molecular analyses have shown that Aub function in GSC biology strongly depends on its role in endogenous mRNA regulation programs. For example, Aub represses expression of the *Cbl* proto-oncogene in GSCs through the binding of its mRNA. Importantly, decreasing *Cbl* gene dose reduces *aub* mutant defects in GSC self-renewal, highlighting the key role of Aub-dependent *Cbl* regulation in Aub function in GSCs (Rojas-Ríos et al., 2017). *Cbl* mRNA regulation by Aub involves recruitment of the CCR4-NOT deadenylation complex, but without poly(A) tail shortening, suggesting repression at the translational level (Rojas-Ríos et al., 2017) (Fig. 3A). Aub iCLIP performed in cultured GSCs has also identified over 1000 mRNAs that are bound to Aub, among which are self-renewal and differentiation factors (Ma et al., 2017). Using reporter transgenes, *dunce* and *bam* mRNAs were shown to be regulated by Aub through their 3'UTRs in GSCs and dividing cysts, respectively. Interestingly, the binding of Aub to both mRNAs exerts a positive effect (Ma et al., 2017), similar to Aub-dependent regulation in the germ plasm of embryos (Dufourt et al., 2017) (Fig. 3A). Although Aub binding to mRNAs in GSCs was proposed to be independent of piRNAs (Ma et al., 2017), this hypothesis is not likely. First, an Aub point mutant unable to bind piRNAs does not rescue *aub* mutant GSC self-renewal defects (Rojas-Ríos et al., 2017); second, Aub crosslink sites in *Cbl* mRNA overlap with piRNA target sites (Rojas-Ríos et al., 2017); third, a *dunce* mutant deleted for the piRNA target site at which ping-pong takes place (Barckmann et al., 2015) shows defects in GSC self-renewal (Ma et al., 2017). These studies demonstrate the crucial role of Aub and piRNAs in the regulation of mRNAs that are involved in self-renewal and differentiation for cell fate transitions in the GSC lineage. However, several questions remain unanswered. For example, how are repression and activation by Aub achieved on different mRNAs in the same cell? How is mRNA cleavage by Aub prevented in these cells? It will be important to address these questions in future studies.

PIWI proteins are also expressed in stem cells outside the gonads. For example, the human PIWI protein Hiwi is expressed in hematopoietic stem cells (Sharma et al., 2001). This finding prompted analyses of PIWI function during hematopoiesis in the mouse model. However, *Miwi2* mutants, as well as *Piwi* gene triple mutants (*Miwi2*, *Mili* and *Miwi*), do not show obvious defects in hematopoiesis, indicating that PIWI proteins are not required in this stem cell lineage under normal conditions (Jacobs et al., 2013; Nolde et al., 2013). A recent study has addressed the role of Piwi in *Drosophila* intestinal stem cells. These cells proliferate in response to an acute stress, such as bacterial infection, to regenerate the intestinal epithelium. This process requires *piwi*, which is activated via JAK/STAT signaling (Sousa-Victor et al., 2017). In addition, Piwi improves intestinal stem cell activity during aging: *piwi* loss of function in these cells induces TE mobilization, DNA damage and apoptosis. This represents a clear example of the requirement of Piwi in the biology of a somatic stem cell lineage, although the molecular mechanisms underlying this function remain to be clarified as the causal relationships between TE silencing and stem cell homeostasis have not been established (Sousa-Victor et al., 2017).

PIWI proteins have also been recognized early as markers of somatic stem cells in non-bilateria species (e.g. sponges,

Ctenophora and Cnidaria) and in primitive bilateria species with high regenerative capacities (Juliano et al., 2011; Ross et al., 2014), suggesting that they are part of an ancestral gene repertoire involved in stemness. For example, piRNAs and PIWI proteins have been analyzed in the cnidarian *Hydra*, in which PIWI proteins are required for regeneration (Bradshaw et al., 2015; Juliano et al., 2014; Krishna et al., 2013; Lim et al., 2014). Knockdown approaches have also revealed the requirement of specific PIWI proteins for whole-body regeneration in bilateria ascidian and planarian species (Palakodeti et al., 2008; Reddien et al., 2005; Rinkevich et al., 2010, 2013). A recent study in the planarian *Dugesia japonica* identified a role of nuclear PIWI in neoblast differentiation and TE repression during differentiation (Shibata et al., 2016). In planaria, only 20–35% of piRNAs map to TEs, indicating piRNA functions in addition to TE regulation (Resch and Palakodeti, 2012; Shibata et al., 2016). Consistent with this, cytoplasmic PIWI proteins are involved in *histone* mRNA localization to neoblast chromatoid bodies in another planarian species (Rouhana et al., 2014).

piRNAs in somatic tissues: roles in brain function and metabolism

Although present at lower levels than in the germline and gonads, piRNAs have been found in various somatic tissues, in particular neurons (Lee et al., 2011; Yan et al., 2011). Neuronal functions of PIWI proteins have been addressed in different species. In *Drosophila*, for example, Aub and Ago3 are expressed in several structures in the adult brain, with lower expression in the mushroom body $\alpha\beta$ neurons that are involved in long-term memory formation. This low expression was proposed to allow TE mobilization in $\alpha\beta$ neurons, producing genomic heterogeneity that might be beneficial for brain function and behavioral variability between individuals (Perrat et al., 2013).

In *Aplysia* (sea slugs), specific piRNAs that are highly expressed in the central nervous system have been analyzed and shown to be involved in long-term synaptic facilitation – a process related to long-term memory – through epigenetic transcriptional repression of the *CREB2* gene (Rajasethupathy et al., 2012). A recent study in *C. elegans* also identified a role of the PIWI protein PRG-1 in neurons. PRG-1 is expressed in adult mechanosensory neurons and is involved in limiting axon regeneration after injury. This PRG-1 function depends on its slicer activity, suggesting the implication of piRNA-dependent post-transcriptional regulation of mRNAs (Kim et al., 2018).

Components of the piRNA pathway, as well as piRNAs themselves, are also found in the adult *Drosophila* fat body (Jones et al., 2016), which is considered to be the functional analog of the mammalian liver. Using *piwi* mutants, the piRNA pathway was shown to be required for essential physiological functions of the fat body. *piwi* mutants show reduced number of fat body lipid droplets, as well as reduced storage of glycogen and triacylglycerides. Strikingly, these defects correlate with shorter lifespan. TEs are deregulated in fat bodies and transposition of *gypsy* was recorded using a fat body-specific *gypsy*-TRAP reporter line. This suggests that defects in fat body physiological functions might involve transposition (Jones et al., 2016). Whether these defects might also implicate endogenous mRNA regulation by piRNAs has not been addressed and awaits further studies. Intriguingly, piRNAs and components of the piRNA pathway are also expressed in the rat liver, with substantial piRNA reprogramming occurring during liver regeneration and carcinogenesis (Rizzo et al., 2014, 2016). This indicates a potential role for piRNAs in liver regeneration and function in mammals. Another recent study using *Miwi2*-deficient

mice identified a role for Miwi2 in lung multiciliated cells in the regulation of innate immunity during pulmonary infection (Wasserman et al., 2017), although the molecular mechanisms underlying this Miwi2 function in the lung remain to be explored.

Importantly, two recent reports exploring the presence of piRNAs and piRNA pathway components in a wide variety of species, including 20 arthropod species (Lewis et al., 2018), and the cnidarian *Nematostella* (Praher et al., 2017), revealed that somatic piRNAs are ubiquitous throughout animal evolution. Moreover, these piRNAs are produced from both TEs and endogenous mRNAs. Analysis of the degradome in *Nematostella* indicates endogenous mRNA cleavage upon targeting by complementary piRNAs. *Nematostella* belongs to Cnidaria, the sister group to Bilateria that includes most animals up to mammals. Therefore, these studies call into question an ancestral-specific role for piRNAs in TE regulation in the germline, and establish that somatic piRNAs and piRNA-dependent regulation of endogenous mRNAs are ancestral and pre-date the separation between Cnidaria and Bilateria (Lewis et al., 2018; Praher et al., 2017).

Conclusions and perspectives

In the last few years, the roles of piRNAs and PIWI proteins in the regulation of gene expression have been extensively analyzed and are now well established. Molecular analyses have revealed that endogenous mRNA regulation by piRNAs is a general process occurring on large pools of mRNAs, and this process is conserved from *C. elegans* to mammals. More recent evolutionary studies indicate that endogenous mRNA targeting by piRNAs is an ancestral function of piRNAs. In addition, these studies also establish that piRNAs act both in germ cells and somatic cells. Therefore, the current view is that the co-existence of somatic and germline piRNA pathways is widespread and that piRNAs are involved in both TE repression and the regulation of gene expression programs.

As in the case of miRNAs, the base pairing of piRNAs with mRNAs can lead to repression through different molecular mechanisms: deadenylation or PIWI-dependent endonucleolytic slicing, depending on the level of complementarity. More surprisingly, mRNA targeting by piRNAs can also lead to mRNA stabilization, through polyadenylation. Whether and how mRNA cleavage is prevented in that case, e.g. using additional proteins, awaits further studies.

At the developmental level, several *in vivo* studies suggest that piRNA-dependent mRNA regulation can fine-tune developmental processes, and acts redundantly with other regulatory mechanisms. For example, the removal of piRNA target sites in *nos* or *dunce* mRNAs leads to intermediate embryonic patterning defects resulting in about 30% embryonic lethality (Barkmann et al., 2015; Rouget et al., 2010). Through their role in endogenous mRNA regulation, piRNAs play conserved functions in key developmental decisions, more specifically linked to sexual reproduction, perhaps in line with their role in the germline to repress TEs. These developmental processes include germ cell specification and development, the balance between GSC self-renewal and differentiation, sex determination and dosage compensation. However, the list of biological processes regulated by piRNA-dependent control of developmental genes is likely to increase rapidly in the near future.

The piRNA pathway also has recognized functions in stemness, in primitive animals with high regenerative capacities, as well as in germline and somatic stem cells in other animals. Consistent with these functions, piRNAs and PIWI proteins are deregulated in a wide range of cancers and other disorders (Han et al., 2017;

Box 3. Outstanding questions

- How does mRNA targeting by piRNAs lead to repression or activation in the same cell, depending on the mRNA? Are additional RNA binding proteins or RNA modifications involved? Are specific proteins required to actively prevent mRNA slicing by PIWI proteins?
- Given the link between PIWI proteins and the translation machinery (Grivna et al., 2006b; Unhavaithaya et al., 2009), and knowing that many components of the piRNA pathway colocalize with translational regulators in RNA granules such as nuage, polar granules and chromatoid bodies (Liu et al., 2011; Meikar et al., 2014; Thomson et al., 2008), do PIWI proteins play a direct role in translation, in addition to their role in the regulation of mRNA stability?
- In *Drosophila* and mice, developmental defects in the germline of PIWI gene mutants depend in part on deregulation of developmental mRNAs. How much derepression of transposition in these mutants cooperates with gene deregulation and contributes to these phenotypes?
- It is known that DNA damage accumulates in piRNA pathway mutants (Chen et al., 2007; Klattenhoff et al., 2007), so does transposition induce DNA damage in these mutants, or does gene deregulation induce DNA damage, which in turn allows transposition?
- mRNA regulation by piRNAs raises several evolutionary questions. The mechanism appears to be conserved throughout evolution, yet, piRNAs are not conserved and their repertoire evolves rapidly, even between populations within a same species. How then can specific regulation be achieved and conserved? In addition, given the high diversity of piRNA sequences and the loose complementarity required to bind mRNAs, how do non-regulated mRNAs escape piRNA binding? Do the mechanisms leading to specific mRNA regulation involve evolutionary constraint of piRNAs or mRNAs? Are additional specific components required?

Ponnusamy et al., 2017; Ross et al., 2014). Indeed, functional studies in specific *Drosophila* models of cancer have identified a role for Piwi and Aub in malignant growth and cancer cell proliferation (Fagegaltier et al., 2016; Janic et al., 2010). Precisely how the piRNA pathway functions in oncogenesis, however, remains to be analyzed. Although this Review highlights important recent progress in our understanding of piRNA functions unrelated to TE regulation, many questions remain unanswered (see Box 3) and represent exciting challenges for future studies in this rapidly expanding field of piRNA biology.

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Competing interests

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