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# Gatekeepers for Piwi–piRNA complexes to enter the nucleus

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RNA silencing pathways are now recognized to participate in essential cellular functions ranging from the regulation of mRNA turnover to the suppression of the activity of potentially deleterious transposable elements (TEs). Piwi-interacting RNAs (piRNAs) are germline-specific, small silencing RNAs that suppress TE activity and maintain genome integrity during germline development. In *Drosophila* ovarian somatic cells, piRNAs are processed from long single-stranded RNAs by a Dicer-independent pathway and are loaded onto Piwi in the cytoplasm. The Piwi–piRNA complexes are then transported into the nucleus to exert TE silencing. This mechanism involves gatekeepers for a functional Piwi–piRNA complex to be imported, which parallels with the *Tetrahymena* Twi1p–scan RNA pathway used to carry out the programmed DNA elimination.

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## Current Opinion in Genetics & Development 2011, 21:1–7

This review comes from a themed issue on  
Differentiation and gene regulation  
Edited by Jessica Treisman and Joel Richter

0959-437X/\$ – see front matter  
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DOI [10.1016/j.gde.2011.05.001](https://doi.org/10.1016/j.gde.2011.05.001)

## Introduction

In RNA silencing pathways, small non-coding RNAs of 20–30 nt function as specificity determinants for the repressive activities of Argonaute-containing effector complexes, termed RNA-induced silencing complexes (RISCs) [1–4]. These recognize their target RNAs by base pairing and promote the inactivation of homologous sequences by a variety of mechanisms in the cytoplasm and the nucleus. RNA interference (RNAi), one form of RNA silencing, was originally described as a silencing phenomenon initiated by exogenous double-stranded RNA (dsRNA) [5], and many classes of small-silencing RNAs including microRNAs (miRNAs) and endogenous small-interfering RNAs (endo-siRNAs) have been shown to be processed by Dicer from their dsRNA precursors. These precursors can be derived from various sources, such as simultaneous sense and antisense transcription of specific genomic loci, fold-back structured transcripts

from repetitive sequences and viral replication intermediates [1–4,6]. We now have a good understanding of the basic biogenesis of small-silencing RNAs, which involves dsRNA precursors and Dicers. Deviations from this paradigm however have been increasingly observed [7,8]. Production of some classes of small-silencing RNAs is Dicer-independent; with current interests also turning to how biogenesis of small-silencing RNAs is physiologically regulated [9,10]. In principle, expression of small-silencing RNAs can be controlled by many mechanisms including transcriptional regulations and epigenetic alterations. However, substantial regulation of mature small-silencing RNA accumulation also occurs post-transcriptionally. Recent studies have shown that many steps in the maturation of small-silencing RNAs are subject to regulation, and that formation and turnover of functional RISCs are also regulated. Therefore, post-transcriptional regulation of small-silencing RNA biogenesis provides an additional level of complexity to RNA-silencing pathways and contributes to the modulation of different biological processes.

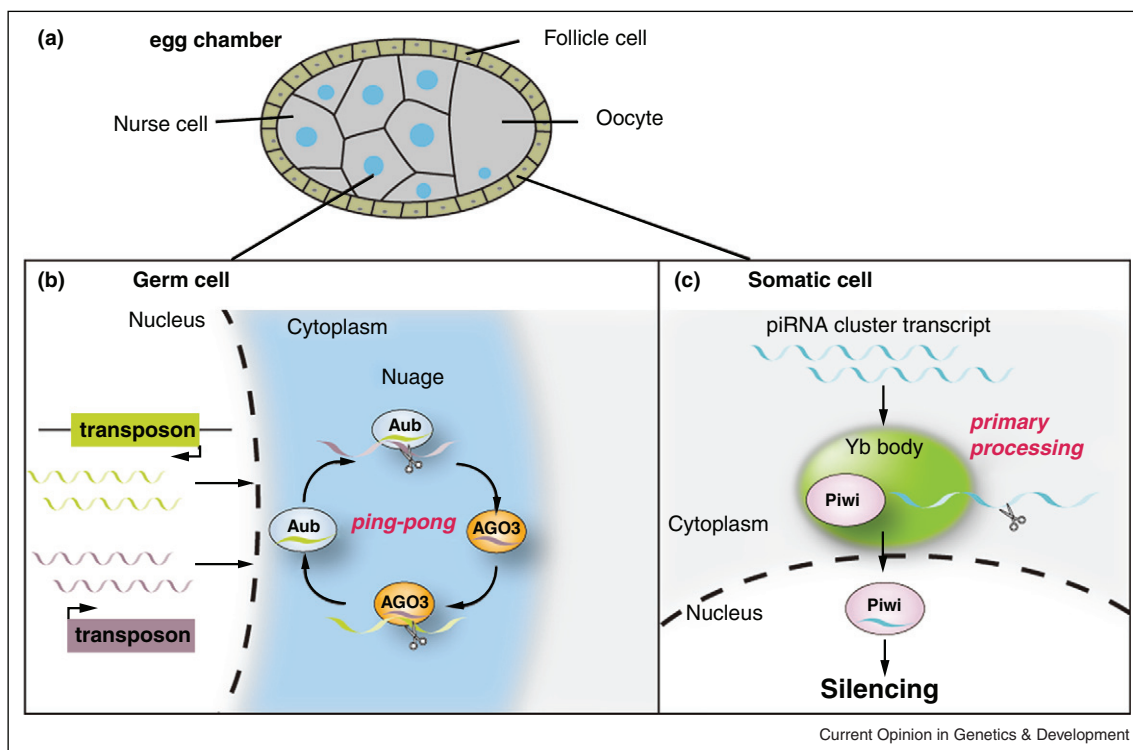
In the *Drosophila* ovary, Piwi-interacting RNAs (piRNAs) arise from repetitive intergenic elements including transposable elements (TEs), via Dicer-independent pathways and function through the PIWI subfamily (AGO3, Aubergine, and Piwi) of Argonautes to ensure silencing of TEs [11,12]. In ovarian somas, Piwi is localized in the nucleus, where it silences TEs; however, piRNA loading occurs in the cytoplasm, which in turn is required for the nuclear entry of Piwi. This suggests a model in which the state of Piwi in ovarian somas is monitored and only a Piwi loaded with mature piRNA is selectively transported into the nucleus to exert TE silencing. Here, we review the recent progress in our understanding of piRNA biogenesis in the *Drosophila* ovary and discuss the nuclear transport system of Piwi–piRNA complexes, which has striking parallels to that of Twi1p–scanRNA complexes in *Tetrahymena*.

## piRNA biogenesis in *Drosophila*

The *Drosophila* genome encodes three PIWI family proteins: AGO3, Aubergine (Aub) and Piwi. Mutations in the PIWI genes lead to defects in germline development and derepression of TEs [13–17]. PIWI subfamily proteins bind to a distinct population of small RNAs, which mainly correspond to TEs, and are now known as piRNAs [18–21]. They are produced via Dicer-independent pathways from long single-stranded RNAs, many of which are transcribed from TE-rich clusters in the genome, and guide TE silencing in *trans* [17–21,22\*,23\*,24]. Two main pathways exist to generate

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Figure 1



The piRNA pathways in the *Drosophila* ovary. The three PIWI proteins — Piwi, Argonaute3 (Ago3), and Aubergine (Aub) — are expressed in *Drosophila* ovaries. (a) Diagram of a mid-stage egg chamber of the *Drosophila* ovary. (b) Aub and Ago3 reside in the nuage, a cytoplasmic structure located in the perinuclear region of nurse cells. Multiple components of the piRNA pathway are enriched in nuage and therefore the ping-pong pathway may operate there. Sense and antisense transcripts from TE loci within the genome of the *Drosophila* ovary are transcribed and do not always form dsRNAs. Slicer activity of Aub and Ago3 is involved in the ping-pong amplification pathway, which amplifies a small initial pool of maternal and primary piRNAs. Amplification only occurs between a pair of piRNAs with a ping-pong signature, thereby avoiding self-directed reactions. Aub associated with antisense piRNA cleaves piRNA precursors in the sense strand. This reaction generates sense piRNAs that are loaded onto Ago3. Ago3 associated with sense piRNA cleaves target antisense transcripts, generating antisense piRNAs that are subsequently loaded into Aub. This reaction also depends on the ongoing presence of a target and therefore steers piRNA production toward active elements. (c) In somatic follicle cells, piRNA cluster transcripts are processed into piRNAs without ping-pong amplification. Mature primary piRNAs are generated in Yb bodies, which are cytoplasmic non-membranous structures found in follicle cells. Processed piRNAs are loaded into Piwi and guide the repression of somatically expressed transposons. Somatic piRNAs corresponding to TEs are almost exclusively derived from the *flamenco* locus. The mechanism(s) by which primary *flamenco* transcripts, approximately 150 kb, are exported to the cytoplasm and selected to be a piRNA precursor remains to be elucidated.

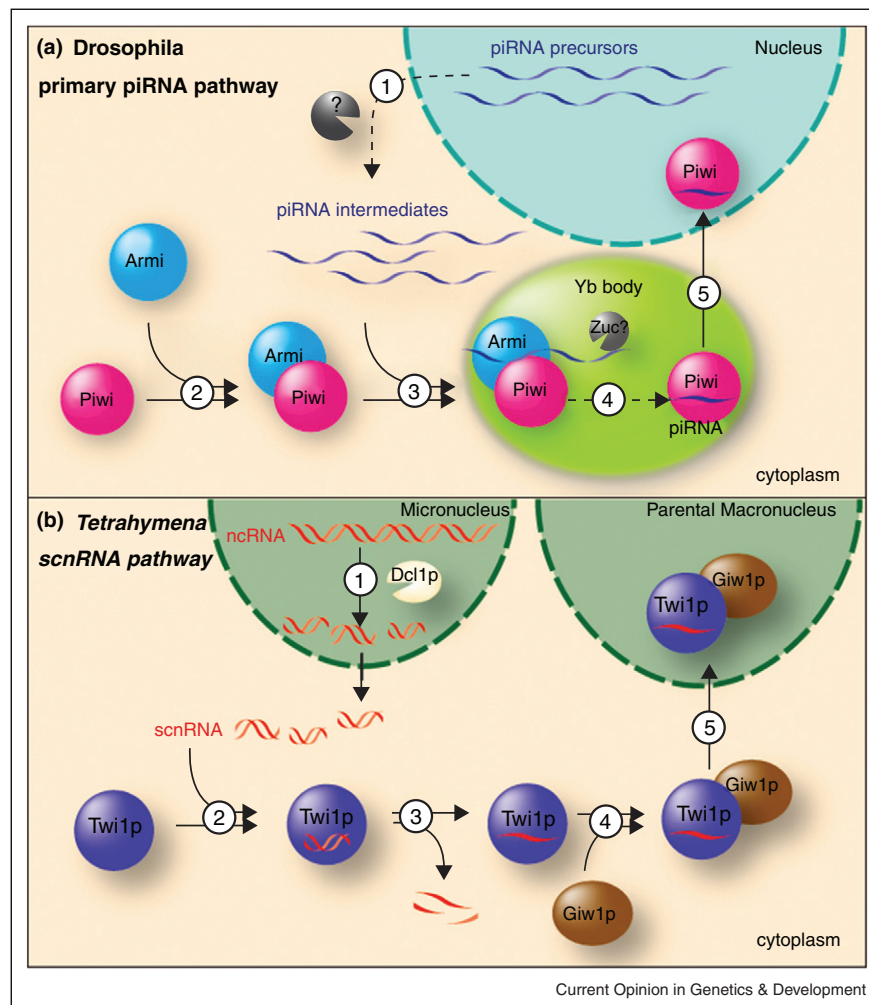
piRNAs in the *Drosophila* ovary [11,12], the ping-pong amplification and the primary pathways (Figure 1). The ping-pong pathway operates specifically in germline cells and mainly engages AGO3 and Aub, both of which are accumulated in nuage [20,21,22\*,23\*]. Nuage are perinuclear structures found at the cytoplasmic face of the nuclear envelope in animal germline cells, where multiple components of the piRNA pathway are enriched (Figure 1b) [21,22\*,23\*,25]. The pathway depends on the endoribonuclease or Slicer activity of AGO3 and Aub, which act catalytically, thereby leading to repeated rounds of piRNA production [20,21]. However, there must be primary piRNAs that initiate this pathway and thus piRNAs produced by the ping-pong pathway are often referred to as secondary piRNAs. It is believed that the ping-pong pathway is primed at least partly by maternally contributed piRNAs [26].

### piRNA biogenesis and nuclear transport of Piwi in ovarian somatic cells

Although AGO3 and Aub are cytoplasmic proteins expressed only in germline cells in the ovaries, Piwi is a nuclear protein expressed in both germline, and somatic cell, such as the follicle cells of the ovaries [18,22\*,23\*]. The mechanism(s) by which Piwi represses TEs in the nucleus remains to be elucidated, but previous studies suggested that Piwi may play a role in heterochromatin formation [27]. Alternatively, Piwi-piRNA complexes may associate with the nuclear RNA turnover machinery, providing them with the target specificity for the degradation of TE transcripts.

Ovarian somatic piRNAs that correspond to TEs are almost exclusively derived from one genomic cluster named the *flamenco* locus, by a pathway independent of

Figure 2



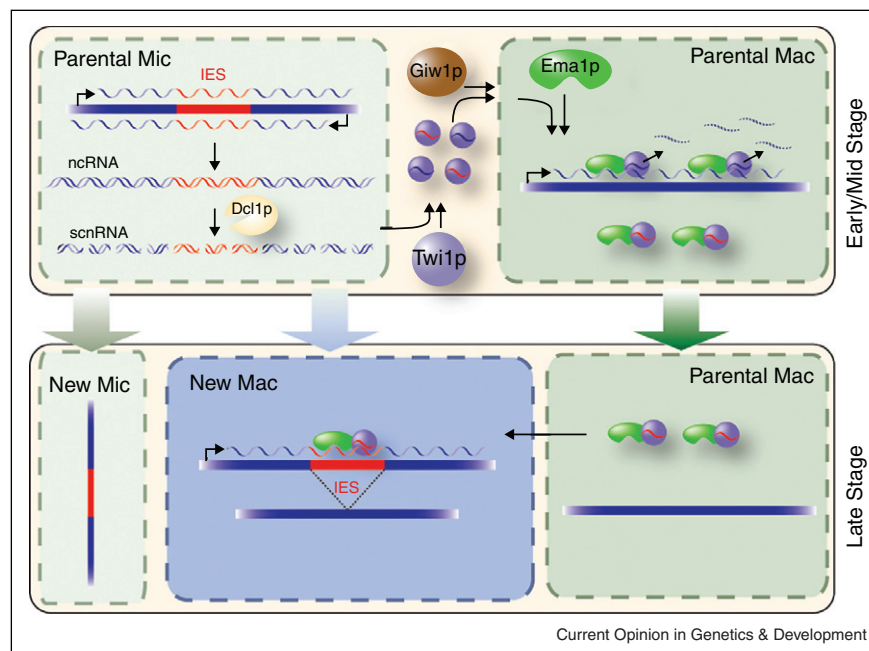
Models of gatekeepers for the nuclear entry of the functional Argonaute–small RNA complexes. **(a)** In *Drosophila melanogaster* primary piRNA pathway, the nuclear localization of Piwi is required for silencing of target transposons. Step 1: piRNA cluster transcripts are processed into piRNA intermediates by unknown mechanisms. Step 2: Armi binds to Piwi and localizes it to Yb bodies. Step 3: piRNA intermediates are assembled into Armi–Piwi complexes at the Yb bodies. Step 4: piRNA intermediates are processed into piRNA and mature piRNA are loaded into Piwi. Zuc, a putative nuclease, is required for primary piRNA processing. Step 5: The functional Piwi–piRNA complex enters the nucleus. **(b)** In *Tetrahymena*, the nuclear localization of Twi1p is required for RNAi-based DNA scanning and elimination. Step 1: In the sexual process of conjugation, the bi-directionally transcribed ncRNAs are processed into scnRNAs by Dcl1p. Step 2: scnRNAs are exported to the cytoplasm, where Twi1p loads scnRNAs. Step 3: The slicer activity of Twi1p removes scnRNA passenger strands. Step 4: Giw1p senses the state of Twi1p–scnRNA complexes and selectively binds with functional Twi1p–scnRNA complexes. Step 5: The Twi1p–scnRNA complex enters the parental macronucleus. Giw1p shows no obvious similarity with any known protein [44\*\*].

the ping-pong cycle and loaded onto Piwi [22\*,23\*,28\*]. This pathway is called the primary piRNA pathway (Figure 1c) [11,12]. The somatic piRNA pathway does not require Slicer activity of Piwi but instead requires the RNA helicase Armitage (Armi), the Tudor domain containing RNA helicase Yb, and the mitochondrial phospholipase D homolog Zucchini (Zuc), as essential factors for primary piRNA biogenesis (Figure 2a) [23\*,28\*,29\*\*,30\*\*,31]. Lack of any of these factors leads to a massive decrease in piRNA levels and TE derepression. The cytoplasmic localization of all three proteins suggests that piRNA biogenesis and loading occurs in the

cytoplasm. Yb is only expressed in the soma, but then accumulates in cytoplasmic foci as ‘Yb bodies’, which localize in close proximity to mitochondria [32]. Armi forms a complex with Piwi and Yb. Without Armi or Yb, Piwi is not loaded with piRNAs and evenly localizes throughout the cytoplasm. Without Zuc, Piwi is not loaded with piRNAs and accumulates at Yb bodies. These findings suggest a model where piRNA precursors are processed in the cytoplasm and formation of a functional Piwi–piRNA complex occurs in Yb bodies before nuclear entry to exert TE silencing. Recent studies have shown that the conformation of Argonaute proteins

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Figure 3



A model of small RNA-directed DNA scanning and elimination in *Tetrahymena*. In the early conjugation stages, the genome of the micronucleus (Mic), including the internal eliminated sequences (IESs), is bi-directionally transcribed, followed by the formation of double-stranded non-coding RNAs (ncRNAs). The ncRNAs are processed by Dicer-like protein Dcl1p to generate scnRNA duplexes. The scnRNAs are exported from the Mic to the cytoplasm, where they are loaded into Twi1p and processed into single-stranded scnRNAs by the slicer activity of Twi1p. Giw1p binds to the Twi1p-scRNA complex and transfers the complex to the parental macronucleus (Mac). Ema1p is required for the interaction between scnRNA and ncRNA in the parental Mac, and induces the degradation of scnRNAs homologous to the DNA sequence. In the later conjugation stages, Twi1p binding with scnRNAs that are not degraded in the parental Mac is transferred to the new developing Mac, followed by the elimination of IESs. In this system, the recognition of germline (with TEs) versus macronuclear specific sequences (without TEs) is achieved by a developmental comparison of parental and developing genomes, guided by small RNAs, in *trans*.

undergoes a dynamic change during loading of small RNAs to accommodate them [33,34]. This conformational change is likely assisted by several of the chaperonins that interact with Argonaute proteins [35–37]. Hsp90 interacts with Piwi and is required for piRNA accumulation [38,39], thereby suggesting that it is a piRNA loading factor. However, a protein(s) that senses the functional Piwi–piRNA complex has not been identified.

### Scan RNA biogenesis and transport in *Tetrahymena*

The nuclear transport of Piwi–piRNA complexes in ovarian somatic cells is likened to that of Twi1p–scRNA complexes in the single-cell ciliate *Tetrahymena*. Like most ciliated protozoa, *Tetrahymena thermophila* possesses two distinctly different types of nuclei in each cell: the larger somatic macronucleus contributes to gene expression; and the smaller germline micronucleus performs reproductive functions [40,41]. Both are descendants of the same zygotic nucleus, but only the micronucleus produces both new micronuclei and macronuclei during sexual reproduction. In the developing new macronucleus, programmed DNA elimination occurs, which restructures the somatic genome to become drastically

different from its germline origin. The approximately 6000 internal eliminated sequences (IESs) mainly consist of repeated sequences that contain TEs. The DNA elimination is mediated by the interaction of germline-produced small RNAs with approximately 28–29 nt, termed scan RNAs (scnRNAs), and transcripts from the macronucleus (Figure 3) [42].

The scnRNA duplexes are processed in the micronucleus by the Dicer-like protein, Dcl1p, from long dsRNAs produced by bidirectional transcription of the micronuclear genome early during conjugation [43]. The duplexes are transported into the cytoplasm, where they are converted into a single-stranded form as they are loaded onto Twi1p, a Piwi protein. Unwinding of the scnRNA duplex is facilitated by slicing the unincorporated strand by Twi1p [44•], similar to the function of AGO2 on siRNA duplexes for the formation of RISCs [45,46]. This results in the production of scnRNAs for all types of sequences including cellular genes and TEs. Because Twi1p is a Piwi family protein, scnRNAs can be considered as the ciliate equivalent of piRNAs. The Twi1p–scRNA complexes are initially channeled to the parental macronucleus, which requires Giw1p (Figure 2b) [44•]. Loading

of scnRNA promotes association of Twi1p with Giw1p and thereby nuclear localization of Twi1p-scnRNA complexes. In the parental macronucleus, it has been proposed that the existing rearranged genome be scanned for homology [42]. The scnRNAs that pair with maternal sequences are removed from the pool of active complexes, most likely, by recognizing nascent long transcripts with the help of the RNA helicase Ema1p [47], while the micronucleus-specific scnRNAs such as IES-specific and TE-specific scnRNAs are enriched. Given the recent findings that extensive pairing between a target RNA and an Argonaute-bound small guide RNA triggers the destabilization of the small RNA [48,49], the removal of scnRNAs by long transcripts is probably achieved by similar pairing-induced degradation of scnRNAs. Finally, Twi1p-scnRNA complexes are exported from the parental macronucleus and re-imported into the developing zygotic macronucleus, where they pair with homologous sequences and induce the elimination of the marked DNA sequences through targeted histone H3K9 methylation [50,51].

### Regulated nuclear transport pathways of Piwi proteins

Nuclear transport of Piwi in *Drosophila* ovarian somatic cells and Twi1p in *Tetrahymena* requires small RNA loading (Figure 2). N-terminally truncated Piwi that is loaded with piRNAs cannot translocate into the nucleus and thus cannot silence TEs [28\*,29\*\*]. There are at least two possible scenarios concerning the nuclear import of Piwi. First, because the N-terminal portion of the protein contains a nuclear localization signal (NLS) that functions in the nongonadal somatic S2 cell line where piRNAs are not produced ([28\*]; K Saito, unpublished observation), the NLS may remain masked by intermolecular folding or interaction with another protein when Piwi is not loaded with piRNAs in ovarian somatic cells. As the conformation of Argonaute proteins changes at a small RNA loading step [33,34], piRNA loading may also induce conformational change of Piwi to dissociate a protein that masks the NLS at the N-terminal part of Piwi. This is akin to the *Schizosaccharomyces pombe* protein Mei2. Accumulation of Mei2 in the nucleus requires a non-coding RNA termed meiRNA [52]. Second, conformational changes following piRNA loading may allow binding of a protein(s) that brings Piwi into the nucleus by providing its specific import signal. This model is related to the *Tetrahymena* Giw1p [44\*\*]. Genetically distinct nuclei in each *Tetrahymena* cell share the same cytoplasm and therefore share the products of most genes that may be equally distributed within the cytoplasm. Binding of Giw1p brings a Twi1p-scnRNA complex into parental macronuclei at the early stages of conjugation. Thus Giw1p not only senses the state of Twi1p but may also supply an initial import signal(s) that channels Twi1p to specific nuclei during sequential transport events. Re-import of Twi1p into developing macronuclei likely

involves a different mechanism. Nuclear transport of Piwi in the ovarian soma depends on the cytoplasmic Yb body, which is not observed in germline cells. Instead, nuclear accumulation of Piwi in germline cells appears to depend on the presence of Aub and AGO3 ([22\*]; A Nagao, unpublished observation). Lack of Aub or AGO3 results in interruption of the ping-pong cycle, leading to the loss of germline piRNAs [22\*,23\*]. Therefore, nuclear transport of Piwi in germline cells may also depend on piRNA loading. How the state of Piwi (with/without piRNA) in germline cells is sensed remains to be elucidated; clearly the sensing system differs from that of the soma, at least concerning the presence and absence of Yb. The mechanisms involved in Piwi's nuclear entry are distinct between the germline and somatic cells in the *Drosophila* ovary.

### Conclusions

The state of the protein to be transported can be modified by interaction with a regulatory protein, which either blocks or promotes association of the machinery that allows for importing of proteins [53]. Given that the nuclear transport of some Argonaute proteins in *Caenorhabditis elegans* [54] and mice [55] is also dependent upon small RNA loading, an important recurring theme is that cells have systems that sense the state of Argonaute/Piwi proteins and transport only functional RISCs into the nucleus. In the future, it will be important to identify factors that recognize only a functional RISC among all other intermediates during RISC maturation. It would also be advantageous to identify the factors that supply specific signals to the RISC for recognition by nuclear transport machineries, and those that act to keep RISC intermediates in the cytoplasm so that nuclear import of premature RISCs is avoided.

### Acknowledgements

We thank Mikiko Siomi, Kuniaki Saito and other members of the Siomi laboratory for comments on the manuscript. This work was supported by MEXT (Ministry of Education, Culture, Sports, Science, and Technology of Japan) grants to HS. HI is supported by the JSPS (Japan Society for the Promotion of Science).

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