Argonaute proteins: key players in RNA silencing

Gyorgy Hutvagner[1] *and Martin J. Simard*[2]

1: Division of Gene Regulation and Expression, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom
Email: g.hutvagner@dundee.ac.uk

2: Laval University Cancer Research Center, Hôtel-Dieu de Québec (CHUQ), Quebec City, Québec G1R 2J6, Canada
Email: Martin.Simard@crhdq.ulaval.ca

ABSTRACT

During the past decade, the small non-coding RNAs have rapidly emerged as important contributors to gene regulation. To achieve their biological roles, these small RNAs require a unique class of proteins called the Argonautes. The discovery and our comprehension of this highly conserved protein family is closely linked to the study of RNA-based gene silencing mechanisms. With their functional domains, the Argonaute proteins can bind small non-coding RNAs and control protein synthesis, affect messenger RNA stability and even participate in the production of a new class of small RNAs.

INTRODUCTION

Despite of the fact that research field on small regulatory RNAs is relatively young, it has already reshaped our understanding of gene regulation by revealing unexpected layers of transcriptional and post-transcriptional gene regulatory mechanisms. Members of a new family of proteins that are involved in RNA silencing mediated by small non-coding RNAs share their names with the great warriors from Greek mythology: the Argonautes.

Argonaute proteins were originally described as being important for plant development [1, 2] and germline stem-cell division in *Drosophila melanogaster* [3]. However, the association of these proteins with small non-coding RNAs, such as in RNA interference (RNAi) and microRNA (miRNA) pathways, has generated significant interest in their study and it has since become apparent that Argonaute proteins are essential for these gene regulatory mechanisms. However, their exact role remained enigmatic for a long time. Many studies have so far attributed roles for Argonaute proteins in maintaining genome integrity, in controlling protein synthesis, RNA stability and the production of a specific set of small non-coding RNAs.

This Review will introduce the origins and the evolution of this family, focus on what is known about the signature and functional domains of Argonaute proteins and describes recent data addressing their functions in animals. Argonaute proteins also have essential roles in yeast and plants in which, among other functions, they are associated with chromosome maintenance and the formation of heterochromatin; however, these topics have been reviewed elsewhere [4,5, 6] and will not be discussed here.

Evolution and diversity of Argonautes

The Argonaute proteins are classified into three paralogous groups: Argonaute-like, that are more similar to the *Arabidopsis thaliana* AGO1, Piwi-like that are more closely related to the *Drosophila melanogaster* PIWI and the recently identified *Caenorhabditis elegans*-specific group 3 Argonautes [7] (Box 1). Argonaute-like and Piwi-like proteins are present in bacteria, archaea and eukaryotes, which implies that both types of proteins have an ancient origin (reviewed in Ref. [8]). However, the number of Argonaute genes present in different species varies. For instance, there are eight Argonaute genes in the human (Four Argonaute-like and four Piwi-like), five in fruit fly genome (two Argonaute-like and three Piwi-like), 10 Argonaute-like in *A. thaliana*, only one Argonaute-like in *Schizosaccharomyces pombe* and at least 26 Argonaute in *C. elegans* (five Argonaute-like, three Piwi-like and eighteen group 3 Argonautes) (Table 1). In some organisms, such as *Trypanosoma cruzi* and *Leishmania major*, the Argonaute proteins may have lost the PAZ domain and retain only a PIWI domain (see below); however, these species are either not sensitive to double-stranded (ds)RNA molecules or these proteins are dispensable for RNAi [9].

Detailed phylogenetic analysis of the Argonaute-like and Piwi-like proteins strongly implies that the last common ancestor of eukaryotes encoded both types of protein. However, lineage-specific loss of either of the paralogues might have occurred over evolution, as plants only encode

Argonaute-like proteins and, for instance, the *Amoebozoa* phylum members have retained only the Piwi-like paralogues. Animals carry representatives of both proteins in their genomes.

Remarkably, Argonautes proteins have undergone a high degree of gene duplication, especially in both plants and metazoans, followed by diversification in their function. The champion of this expansion is *C. elegans*, with 26 distinct Argonaute proteins (see below). On the other hand, there are examples of simplification and loss of Argonaute proteins in taxonomically diverged organisms. For instance, *S. pombe* has retained one Argonaute-like protein, which functions in diverse processes such as heterochromatin silencing and post-transcriptional gene silencing (PTGS) [10-12]. Furthermore, *S. cerevisiae* and some parasitic organisms such as *T. cruzi* and *L. major* may have independently lost their Argonautes together with the entire RNAi machinery [9].

Comparative in silico analysis of the evolution of the key components of the RNAi machinery implies that the last common ancestor of eukaryotes already had at least two distinct RNAi mechanisms, based on the prediction that this organism contained at least one Argonaute-like and one Piwi-like protein. Piwi-like protein most likely, might have localized to the nucleus and been an effector of mechanisms related to transcriptional silencing such as transposon silencing, silencing of repetitive elements that result in heterochromatization, whereas the Argonaute-like paralog was responsible for the regulation of translation by targeting mRNAs in the cytoplasm, as these two types of silencing phenomenon are present in all organisms that can use dsRNA to regulate gene expression [8]. Gene duplication of Argonautes probably resulted in the diversification of RNAi-like mechanism in which the effector proteins probably specialized to bind distinct small RNA species and/or interact with diverse protein complexes with different regulatory potentials.

Biochemical analysis of eubacterial and archean Argonaute proteins indicates that the original function of this protein family was similar to the function of members of the ribonuclease (RNase) H family of endonucleases; an endonuclease that uses DNA as a template to target RNA

molecules [13, 14]. However, over evolution Argonautes specialized to use single-stranded (ss)RNA rather than DNA as a template to target RNA. In animals, some Argonautes have lost their catalytic activity, and participate in a gene regulation mechanism that does not require RNA cleavage (see catalysis-independent Argonaute activity section below).

Functional domains of Argonautes

Argonautes consist of four distinct domains: the N-terminal, PAZ, Mid and PIWI domain (Figure 1). Eukaryotic Argonaute proteins that function in gene regulatory mechanisms mediated by small RNAs always contain these domains.

The long-awaited explanation for the function of Argonaute proteins started to be resolved with the help of crystallographic studies. These studies revealed that the PIWI domain of Argonautes folds in a similar manner to the catalytic domain of RNase H enzyme family, which strongly suggested that Argonautes function as endonucleases [15]. Interestingly, the eubacterial *Aquifex aeolicus* and the archean *Pyrococcus furiosus* Argonaute proteins can bind single-stranded (ss)DNA [13, 14] and *A. aeolicus* efficiently cleaves DNA–RNA hybrids, which indeed implies an RNase H-like role for the ancient Argonautes [13, 14].

The PAZ domain. The PAZ domain is only found in Dicer and Argonaute proteins, two protein families with key roles in RNAi mechanisms. The PAZ domain consists of two subdomains, one of which displays OB-like (oligonucleotide-oligosaccharide binding) folding, which indicates that the PAZ motif might bind single-stranded nucleic acids [16, 17, 18]. Crystallography combined with biochemical approaches indeed proved that the PAZ domain binds to ssRNAs with low affinity in a sequence-independent manner [19, 20].

The remarkable nature of the PAZ domain is that it can recognize the 3´ ends of ssRNAs. Both miRNAs and distinct types of small interfering (si)RNAs are trimmed by the sequential action

of RNase III enzymes (Drosha and Dicer in animals or Dicer only in yeast and plants), both of which characteristically leave two 3´overhangs on the processed product. Therefore, the PAZ domain could initially distinguished these small regulatory RNAs from degraded RNAs derived from non-related pathways by binding to their characteristic 3' overhangs.

The PIWI domain. The structure of the full-length archean and eubacterial Argonautes and the archean *Archeoglobus fulgidus* Piwi protein, which lacks the N-terminal domain and the PAZ domain, revealed that the PIWI domain has an RNase H-like fold [13-15, 21]. As mentioned above, RNase H-like enzymes cleave RNA using a DNA template and this catalysis requires a conserved Asp-Asp-Glu/Asp motif in the catalytic centre and binding of two divalent metal ions by the ribonuclease. Cleavage-competent Argonaute proteins have a slightly more degenerate catalytic centre (Asp-Asp-Asp/Glu/His/Lys) and they require the binding of a divalent cation for activity (reviewed in Ref. [22]). Their cleavage products contain 3´-OH and 5´-phosphate, which is also a characteristic feature of RNase H-like processing [23, 24].

Additional important insights into target recognition and activity were gleaned from structural studies in which *A. fulgidus* Piwi and the eubacterial *A. aeolicus* Argonaute were crystallized in the presence of either ssRNA or siRNA-like molecules [14, 25]. These studies reconfirmed earlier works indicating that the 5´ phosphate of an siRNA or a miRNA is a key element of their functionality [26]. The 5´ phosphate is anchored, via a divalent cation, at the interface between the PIWI and the Mid domain [14, 25]. Apart from its function to anchor the 5' phosphate, the Mid domain of metazoan Argonautes that function in the miRNA pathway contain a portion, known as the MC domain, that has striking homology to the cap-binding motif of the translation initiator factor eIF4E. Indeed, the MC domain can bind to the cap and it is required for efficient regulation of translation [27]. The above-mentioned structural studies show that the first nucleotide of the guiding strand of a small RNA is separated from the targeted RNA. This is in agreement with reports that

imply that the very 5´ end nucleotide of siRNAs and miRNAs is not necessarily involved in the recognition of the substrate [28-30]. Moreover, the results of structural studies explain why the small RNA-mediated cleavage of the target RNA always occurs at a fixed place (as established by previous biochemical studies), as the catalytic motif of the ribonuclease is positioned in front of the scissile phosphate between the 10th and the 11th nucleotide of the guiding strand (counting from the anchored 5´ end) [29, 31].

A study in which recombinant proteins were used showed that the minimal RNA-induced silencing complex (RISC) contains a cleavage-competent Argonaute or the PIWI domain of an Argonaute plus a bound small RNA [32]. The endonuclease activity of the Argonaute and Piwi proteins in fission yeast, fungus, plants, flies and mammals is essential for the function of RISC. Argonautes have been shown to participate in the maturation of siRNAs by eliminating the non-active siRNA strand [33-35] and initiating sequence-specific cleavage of the target RNAs [36]. In addition, the cleavage activity of Piwi proteins is most likely required for the maturation of repeat-associated small interfering (rasi)RNAs and Piwi-interacting (pi)RNAs in flies and mammals [37, 38]. The presence of an intact PIWI-domain catalytic centre, however, only partially explains the cleavage activity of the Argonautes. For instance, human Ago3 is incapable of mediating cleavage if it is charged with miRNAs, despite it having a canonical active site, which implies a requirement for binding to specific, as-yet-undiscovered, small RNA(s) or additional co-factors [39-41].

Catalysis-independent Argonaute activity. The catalytic activity of the PIWI domain is clearly important for some Argonaute proteins, but other members of this family, such as human Ago1, Ago4, one of the human Piwi HIWI2/PIWIL4, and most of the group 3 Argonautes of *C. elegans*, have diverged in their catalytic motif to a degree that probably impairs their endonuclease activity [22]. Importantly, not all known small-RNA-mediated gene regulation requires the catalytic activity of the Argonautes. For instance, the majority of miRNA-mediated gene repression in

animals, in contrast to plant miRNAs, does not involve sequence-specific cleavage (called slicing activity), even if the miRNAs are incorporated into Argonautes that maintain the catalytic activity (slicing-competent Argonautes) — this is because miRNAs share only restricted complementarity to their target RNAs and this is insufficient for sequence-specific cleavage.

Argonautes are involved in distinct steps of small RNA maturation and small RNA-mediated gene repression that very likely require interactions with diverse protein complexes. Indeed, the comprehensive cytoplasmic human Ago1 and Ago2 proteomes, affinity purified with overexpressed tagged proteins, have been recently reported [42]. This study showed that human Ago1/2 interact a variety of proteins in three distinct sized complexes. The majority of these proteins are RNA binding proteins that are involved in distinct steps of RNA processing, maturation, transport and the regulation of RNA stability and translation. Interestingly, some of the interactors have function in other small RNAs such as small nuclear (sn)RNAs and small nucleolar (sno)RNAs biogenesis. Some of these interactions are likely to be mediated by RNAs, but some proteins may bind directly to Argonautes or associate with them via other protein interactors [42]. It has already been established that Argonautes can directly bind to other proteins. Both Dicer and Argonaute proteins participate in the selection of the active strand of siRNAs and miRNAs, and it was demonstrated that the PIWI box, a motif located within the PIWI domain, of human Argonautes binds to one of the RNase III domains of Dicer [43]. The PIWI domain of the fly AGO1 directly interacts with GW182, a protein characteristic of the cytoplasmic processing body (P-body). GW182 has also a role in the miRNA-mediated gene regulation and which could function downstream of AGO1 in flies [44]. This latest interaction represents a unique way in which Argonautes interact with other proteins. The part of the PIWI domain that accommodate the 5´ phosphate of the guiding strand of an siRNA also bind to an at least 22 long peptide, called the Ago hook, originally recognized in the *S. pombe* Argonaute interacting protein Tas3 [45]. This peptide contains WG/GW repeats and the tryptophan residues are

absolutely required for the interaction. Interestingly, this type of amino acid repeats are found in many unrelated Argonaute interactor proteins such as the large subunit of plant Polymerase IV (NRPD1b), the orthologues of the GW182 protein family in metazoan and the yeast Tas3 [45, 46]. In NRPD1b and GW182 proteins, the GW/WG motifs are found in many copies and they may provide a scaffolding platform for binding multiple Argonautes in order to assembly the regulatory complex. Since in vitro experiments showed that the Ago hook peptide can relieve miRNA-mediated gene repression in fly extract, despite that the PIWI domain is able to mutually bind to either the small RNA or the peptide, it is very likely that this motif also participate in the small RNA-mediated gene regulation [45].

Argonautes in RNA silencing pathways

Into the cell, small non-coding RNA species bound by Argonaute proteins are either exogenously supplied by scientists or viruses or endogenously produced by various molecular processes (Box 2). Once processed, the loading of small RNAs into the Argonaute proteins requires various protein complexes; for two of the most studied pathways, the RNAi and miRNA pathways, and this assembly process is shown in Figure 2. The selection of the strand to remain bound to the Argonaute is guided by the thermodynamic stability of the 5' ends of the small RNA, in a process referred to as the asymmetry rule. In general, the RNA molecule from the double-stranded precursor that has the less stable 5' end will be incorporated into the RISC complex, whereas the other strand will be destroyed [47, 48].

The processing and loading of regulatory small RNAs into distinct Argonaute complexes show specialization in many organisms. For instance, in *A. thaliana* different Dicer and Argonaute complexes are responsible for processing and binding distinct small RNA species such as miRNAs, trans-acting small interfering (tasi)RNAs and repeat-associated small interfering (rasi)RNAs

(reviewed in Ref. [4]). In flies, Dcr-1 and Loquacious are required for miRNA biogenesis [49-51], whereas Dcr-2 and R2D2 are responsible for the production of siRNA from long, perfectly paired dsRNA [52, 53]. Recent studies revealed that the structure of the 'diced' siRNA and miRNA intermediate duplexes determines their partitioning into Ago1 or/and Ago2 complexes in *D. melanogaster* [54, 55], and influences the selection of RDE-1, ALG-1 or ALG-2 proteins in *C. elegans* ([56], Jannot G., Boisvert M.E., Banville I.H. and M.J.S., unpublished observations).

In the siRNA pathway, the binding orientation of a heterodimer of Dcr-2 and R2D2 on the siRNA duplex molecule is important for determining which one of the two siRNA strands is loaded into the Argonaute complex [53, 57]. R2D2 will bind to the more stable end, whereas Dcr-2 is recruited to the less stable end (Figure 2). It is proposed that this complex (called the RISC-loading complex, or RLC) will recruit an Argonaute complex, referred to as the holo-RISC, by an interaction between Dcr-2 and Ago2 [58]. Once Ago2 is associated with the siRNA duplex, the Argonaute will cleave the non-active siRNA strand (the passenger strand) and thus, initiate unwinding and release to generate the active RISC complex of Argonaute and the small RNA [34]. Although the cellular factor that confers unwinding activity has not yet been uncovered, biochemical studies have clearly demonstrated the essential contribution of Argonaute in this process [53, 59].

Argonaute proteins and small regulatory RNAs in nematodes. The discovery of the first Argonaute gene associated with the RNAi response, *rde-1* [60], led to the subsequent discovery of the existence of 26 Argonaute-family members in *C. elegans* (Box 1). Seminal work to uncover the function of these genes led to the discovery of ALG-1 and ALG-2, which are essential for the miRNA pathway [61]. Their loss-of-function generates problems in the timing of the animal development also called heterochronic phenotypes — hallmarks of miRNA defects in *C. elegans* [62, 63]. An exhaustive study of the remaining members of the Argonaute family in the nematode demonstrates the importance of these genes in various RNA silencing pathways (Table 1; Ref. [7]).

Whereas RDE-1 is required only for RNAi mediated by exogenously supplied dsRNA triggers (exogenous RNAi pathway) and ERGO-1, CSR-1, are essential for RNAi initiated by dsRNA molecules generated within the cell (endogenous RNAi pathway), others Argonautes function in both RNAi pathways [7].

Interestingly, SAGO-1, SAGO-2, PPW-1, PPW-2, C16C10.3 and F58G1.1, the Argonaute proteins that are important for both exogenous and endogenous RNAi, bind another class of small RNAs: the secondary siRNAs that are a distinct class of siRNAs (Figure 3). The production of these RNA species is initiated by the first Argonaute (RDE-1 and most likely ERGO-1 and CSR-1)–siRNA complex, which recognizes the targeted mRNA and induces the synthesis of an antisense strand by RNA-dependent RNA polymerases (RdRPs) that thus will lead to their production (Figure 3). The sequential requirement of Argonaute proteins seems to be associated with their capacity for RNA cleavage. Sequence alignment in the three regions of Argonaute proteins that have similarity to the catalytic centre of RNase H indicates that the Argonaute proteins associated with the trigger-derived siRNAs contains the specific residues for cleavage, whereas others associated with the secondary siRNAs lack these residues [7]. Interestingly, it has been recently observed that the amplified siRNAs in *C. elegans* have two or three phosphate residues at their 5' ends [64, 65], whereas Dicer-derived siRNAs carry a single phosphate [26, 31]. Therefore, the specific binding of an Argonaute protein to either primary or secondary siRNAs may be guided by the number of phosphates found at the 5' ends of small RNAs that reflect the machinery that generate these RNA species.

Biological outcomes

Seminal studies in *C. elegans* and plants have uncovered the important role carried out by Argonaute proteins in RNA silencing pathways. At the same time, studies in fly and mammalian

systems have increased our understanding of the molecular roles of the Argonaute proteins in these biological processes.

Argonautes interfere with translation. One of the most extensively studied functions of Argonautes is their role in regulating translation that is in association with miRNA-mediated gene regulation. For a long time, Argonautes were believed to only be involved in translational repression; however, a recent study has revealed that Argonautes can be a part of a protein complex that enhances the translation of an AU-rich-element-regulated transcript in serum-starved cells [66]. In the past two years, another dogma — namely, that miRNAs regulate translation at the elongation step without influencing the stability of the target RNA — has been challenged [67].

In fact, it seems that Argonautes, in a complex with miRNAs, can influence translation in many distinct ways. Increasing *in vitro* and *in vivo* evidence suggests that Argonautes might inhibit the translation of miRNA-targeted RNAs at the initiation step. The studies supporting this mechanism showed that the great majority of the miRNA-mediated gene regulation requires the presence of the canonical cap and poly(A) tail on the targeted RNA [68, 69]. Furthermore, tethering translation initiation factors on the miRNA-targeted RNA abrogates miRNA function and certain internal ribosome entry site (IRES) elements render the RNA resistant to miRNA-mediated repression [69, 70].

According to recent studies, translation initiation can be prevented in at least two distinct ways. In cell-free extract from fly, miRNA inhibits the formation of the translationally active 80*S* ribosome by inhibiting the assembly of the 43*S* initiation complex [71]. Argonautes can bind to the cap potentially through its MC domain, and mutations in this cap-binding domain impair their function in translation repression [27]. Therefore, competition between Argonaute proteins and translational initiation factors for cap binding might explain the inhibition of the assembly of the 43*S* complex [27]. In addition, miRNA-mediated repression of translation requires eIF6, the anti-dissociation factor

that prevents the assembly of the 80*S* ribosomes [72]. As eIF6 binds to the 60*S* ribosomal subunit, this type of inhibition of translational initiation is clearly distinct from inhibition of the assembly of the 43*S* initiation complex on the cap.

Several lines of evidence also support the notion that miRNAs in complex with Argonautes could alternatively inhibit translation after initiation. miRNAs and their targets have been shown to co-sediment with actively translating polyribosomes, which implies that repression occurs after initiation and might result in ribosomes falling off the polypeptide chain, or the synthesized polypeptide chain being rapidly degraded [67, 73-77].

It might seem strange that miRNA-loaded Argonaute proteins can inhibit translation at different stages, but we think it is plausible that these proteins interfere with translation in diverse ways, providing a fail-safe mechanism to reduce or abolish harmful protein expression. But the question remains: how do Argonaute proteins mediate this process? A clever set of experiments has demonstrated that, in the absence of miRNAs, tethering functional human Argonautes to the target RNA can induce gene silencing [78]. These data indicate that the small RNA molecules in the complex function only as a sequence-specific tag to deposit the machinery to the mRNA. It then becomes important to characterize the proteins that associate with Argonautes and/or miRNA-targeted RNAs to mediate the silencing.

The cap-binding capacity of the Argonautes could explain the inhibition of the assembly of the 43*S* complex and it also clarifies why increasing the number of miRNA-binding sites on a target elicit more prominent regulation, as a higher number of Argonaute proteins associated with the target result in a more dramatic cap-binding potential [27, 28]. However, it is very likely that other types of translational interference require additional protein interactors. For instance, in the fly, tethering GW182, a protein that directly interacts with fly AGO1, to mRNA in an AGO1 knockdown

background recapitulates translational repression. This observation indicates that other events, downstream of Argonautes, might regulate protein synthesis [44].

On the other hand, it has recently been observed that miRNA-mediated gene regulation could be relieved in specific biological conditions. Under specific stress, miRNA-mediated gene repression could be reversed by HuR, a protein that recognizes AU-rich regulatory elements on the miRNA-targeted untranslated region (UTR) [79], which indicates that miRNA-mediated gene regulation is not irreversible.

Argonautes and RNA stability. Argonaute proteins complexed with miRNAs can alter the stability of targeted RNA without initiating sequence-specific cleavage, especially in metazoans, in which the complementarity of most miRNAs to their target is insufficient to induce endonuclease activity [80-84]. There is evidence that this RNA destabilization is independent of translation and requires the canonical cap and poly(A) tail, which suggests that the RNA degradation is executed by the machinery that governs the 5′→-3′ decay after deadenylation-dependent decapping [81, 85]. A comprehensive study in *D. melanogaster* has shown that the miRNA-mediated RNA decay requires GW182, which recruits the CCR4–NOT deadenylase and DCP1–DCP2 decapping complexes, and that these complexes are responsible for the decay of the miRNA-targeted transcripts [44].

The question is whether RNA degradation is a consequence of translational repression or whether they are two independent mechanisms. Increasing evidence indicates that miRNA-mediated target RNA decay and the repression of translation can be uncoupled. For instance, not every miRNA-targeted RNA shows destabilization at the steady-state level [44, 68-70, 77, 79]. Furthermore, it was shown that inhibition of the pathway that degrades the miRNA-targeted transcripts generated more stable RNA but it did not relieve translational repression, which strongly supports the idea that the two events are independent of each other [44]. It was demonstrated in fly that the level of RNA decay could vary in individual miRNA–target-RNA interactions, which suggests that the interaction

between a miRNA-protein associated complex and a specific mRNA can regulates the level of decay [44]. Factors that are suggested to be important are those responsible for regulating the stability and the turnover rate of the targeted RNA and/or the miRNA–target complex [44, 86]. It is very likely that additional proteins that are either associated with the core Argonaute–miRNA complex or bound to the targeted RNA could determine the degree of stability of the translationally repressed miRNA targets. Indeed, AU-rich element-binding motifs and proteins have already been demonstrated to interfere with miRNA-mediated gene regulation [66, 79, 87].

Piwi-like proteins and germ line maintenance. Piwi-like proteins, which are found in metazoans, are important for the production and function of germline stem cells [88, 89, 3, 7, 90-92]. These proteins have recently been found to be associated with a new class of small RNAs called the Piwi-interacting (pi)RNAs, which are specifically expressed in germ cells [93-97] and required to silence mobile elements and thereby maintain genome integrity [38, 98-100]. In contrast to other Argonaute-associated small RNAs, piRNAs are slightly longer (24–30 nucleotides long) and their production does not require the RNase III gene-family members Drosha and Dicer [98, 100]. The generation of piRNAs relies instead on the endonuclease activity of Piwi-like proteins (Box 2). It has recently been observed in *D. melanogaster* that initial cleavage of an Ago3–piRNA precursor by a complex of Aubergine and piRNA induces the exponential production of piRNAs [37, 38]. It is still not known how the primary source of piRNAs is generated or how the 3´ end of a piRNA is defined, although recent genetic data from flies suggest that additional endonucleases and exonucleases can participate in these events [101].

Recent data obtained by Lin, Elgin and colleagues with the fly system has brought new insights on Piwi-like proteins functions [102]. They observed that the N-terminal domain of *Drosophila* PIWI, can interact with heterochromatin protein 1a (HP1a) dimer, a non histone chromosomal protein that plays important roles in chromosomal biology and gene silencing

(reviewed in ref. [103]). HP1a and PIWI colocalize in pericentric heterochromatin regions and this cellular localization appears to be RNA dependent. More importantly, they also demonstrated that the interaction with HP1a is important for PIWI epigenetic function since mutations into the interaction domain that abrogate HP1a binding fail to rescue silencing in PIWI-depleted animals. This new observation supports for the first time that Piwi-like proteins in the metazoans may be implicated in heterochromatin formation in a similar manner as Ago1 in fission yeast where the protein targets histone methylation to create binding sites for the HP1 homolog Swi6 [11,104]. Additionally, *Drosophila* PIWI protein can also promote production of piRNAs by increasing transcription of piRNAs loci located in subtelomeric regions [105]. Based on these two new studies, we can envision that Piwi-like proteins will contribute to the initial production of piRNAs and then bind to these newly synthesized small RNAs in order to silence specific chromosomal regions (i.e. pericentromeric regions) through the interaction with HP1. These recent works start to reveal how Piwi-like proteins can be implicated in germ line maintenance by regulating chromosomal states of stem cells genome.

CONCLUSIONS AND FUTURE DIRECTIONS

Since the discovery of the first Argonaute gene in *Arabidopsis thaliana* only ten years ago, members of this family have rapidly emerged as key components of new gene regulation pathways that involve small non-coding RNAs. A significant number of recent studies using a variety of biological systems have started to reveal the impressive biological capacities of the Argonaute protein family.

Biochemical studies of Argonaute proteins from different species have provided a better understanding of the molecular features that define the enzymatic activity of the PIWI domain, and the capacity of the PAZ domain and Mid domain to interact with small RNA molecules and proteins involved in translation. Model organisms such as *A. thaliana*, *C. elegans* and *D. melanogaster* have

helped to uncover the functional diversity of the roles of Argonaute proteins in many developmental cues as well as during cell proliferation and differentiation. Studies performed with model organisms and mammalian cell culture systems have started to shed light on how Argonaute proteins, in association with small non-coding RNA pathways, can control protein production and the stability of targeted mRNAs and even directly contribute to the production of small RNAs.

In the next years, the real challenge will be to determine how Argonaute proteins regulate gene function. For this, it will become essential to discover their biological partners, understand their tissue and developmental specificities and their capacity to precisely interact with various small RNA species. It will also be interesting to identify molecular features such as post-translational modifications that modulate the Argonaute proteins for such an extreme functional diversification found in metazoans and especially in the nematode *C. elegans*.

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Display items

Boxes

Box 1 | **The origin of the Argonautes**

The term Argonaute was originally used by Bohmert and collaborators to describe a mutant of *Arabidopsis thaliana* (*AGO1*) in which the morphology of the leaves resembled a small squid: the

'greater argonaut' or *Argonauta argo* [2]. Sequence analysis of a new gene required for plant development, *ZWILLE,* has highlighted the presence of a highly conserved 70-amino-acid sequence that is found in *AGO1* as well as in certain proteins in nematodes and humans; the PAZ domain [1].

Genetic studies in fruit fly identified *piwi* (for *P*-element-*i*nduced *wi*mpy testis), a gene essential for germline stem cell division [3] and the *piwi* box, a 40-amino-acid sequence located in the C terminus of the *piwi* protein, in the *A. thaliana* proteins *AGO1* and *ZWILLE*, in one human protein HIWI and in many *Caenorhabditis elegans* proteins [91]. This study led to the first characterization of the PIWI domain. In 2000, the PIWI domain was redefined as a 300-amino-acid region and demonstrated to be present in prokaryotes [106]. This same study also identified a region of similarity between the central portion of the fly Piwi protein, which is common to all Argonautes, and the carpel factory protein from *A. thaliana*, an important gene for plant development [107]. This 110-amino-acid region was designated the PAZ domain, after three proteins sharing this domain: *PIWI*, *AGO1* and *ZWILLE*.

The figure shows the phylogenetic relationship of the Argonaute proteins. The Argonaute-like group found in plants, animals and fungi are indicated in black. The Argonaute clade in green represents the Piwi-like group. The *C. elegans*-specific group 3 Argonaute proteins are indicated in red. *C. elegans* M03D4.6 and C06A1.4 are most likely pseudogenes. Argonaute genes are also found in prokaryotes (not shown). Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; and Sp, *Schizosaccharomyces pombe*. Figure modified with permission from Ref. [7].

Box 2 | **Diverse sources of small RNA molecules**

Various viruses and experimental methods can exogenously introduce into the cell micro (mi)RNAs, small interfering (si)RNAs or double-stranded (ds)RNA molecules that will be processed into small RNAs by Dicer, an RNAse III-type enzyme. Endogenously, small RNAs can be generated by

different sets of enzymatic activities. As demonstrated in *Drosophila*, Piwi interacting (pi)RNAs are produced by a sequential action of two Piwi-like proteins. The Ago3 protein bound mainly piRNAs derived from sense strand of retrotransposons while piRNAs derive from antisense strand is associated to Aubergine (Aub) protein. Interestingly, it has been observed that the first 10 nucleotides of Ago3-interacting piRNAs can be complementary to the first 10 nucleotides of Aubergine-interacting piRNAs. In this model, called the "ping-pong" model [37, 38], the cleavage induces by the Ago3-piRNAs complex specifies the 5' ends of Aubergine-associated piRNAs. Therefore, the Aubergine-piRNAs complex can then produce the 5' ends of Ago3-associated piRNAs. Subsequently, a methyl group is added to their 3'-ends (gray circle)[108] by a methyltransferase called Pimet/DmHen1 [109, 110]. Genes encoding miRNAs are first transcribed mainly by RNA polymerase type II [111] to produce pri-miRNA (few miRNAs have been reported to be products of RNA polymerase type III [112]). After being trimmed by the RNase III Drosha complex [113] or processed by Dicer in plants (or, in some cases, intronic miRNAs (miRtrons) in flies and nematodes bypass Drosha [114, 115]), the pre-miRNA is exported into the cytoplasm through exportin-5 [116] to then be processed by the Dicer complex [61, 117, 118]. Endogenously, siRNAs originated from bi-directional transcription of specific chromosomal regions (centromeres and mating type locus) or aberrant production of dsRNA from repetitive regions once cleaved by Dicer. In plants and nematodes, a significant portion of siRNAs is also produced from the activity of RNA-dependent RNA polymerases or RdRPs (not shown; see Figure 3).

Figures

Figure 1 | **Structural features of Argonaute proteins. a** | Linear view of an Argonaute protein (human AGO2 is represented as an example). The PAZ domain (yellow), which is important for

small RNA association along with the PIWI domain, is situated near the N terminus of the protein (white). The PIWI domain (purple) of cleavage-competent Argonaute proteins contains the catalytic residues DDH (highlighted in red), which are essential for cleavage. The cap-binding-like domain (MC; green) is found within the Mid domain (light green); the region flanked by the PAZ and the PIWI domains. Coloured residues represent those conserved in the cap-binding factor eIF4E. **b** | Crystal structure of the Argonaute protein from *Pyrococcus furiosus*. The small interfering RNA (shown in purple) and the mRNA (in light blue) have been superimposed in the model. Active residues of the PIWI domain are shown in red. Reproduced with permission from Ref. [15]. An alternative model have been produced from a structure of *Aquifex aeolicus* Argonaute crystallized in the presence of single-stranded 8 nucleotides long RNA [13, 14].

Figure 2 | **Assembly of the Argonaute complex.** Inside the cell, a double-stranded (ds)RNA duplex is bound by a recognition complex that contains a Dicer-family member (DCR) and a dsRNA-binding protein (green). In *Drosophila melanogaster*, the dsRNA-binding protein Loquacious forms the miRISC loading complex (in the microRNA pathway; right) with Dcr-1, whereas, in the RNA interference (RNAi) pathway, (left) Dcr-2 and R2D2 are important for recruiting the Argonaute protein. Once the Argonaute is associated with the small RNA duplex, the enzymatic activity conferred by the PIWI domain cleaves (star) only the passenger strand (black strand) of the siRNA duplex (RNAi pathway). Mismatches found into the miRNA duplex interfere with cleavage although, in some situations, the passenger strand might be cleaved if the RNA duplex is fully paired. RNA strand separation and incorporation into the Argonaute complex is guided by the strength of the base-pairing at the 5' ends of the duplex, the 'Asymmetry rule' (for further information on the free energy of a dsRNA duplex, please refer to [119]). In this example, the 'easiest' 5' end to unwind is highlighted in yellow. Once unwound, the siRNA or miRNA will associate with

the Argonaute protein (and probably other cellular factors) to form the RISC or miRISC complex, respectively. However, because of their unique structure of the intermediate RNA duplex, some miRNAs could be sorted into both Ago1 and Ago2 complexes in *D. melanogaster* (pathway indicated by the blue arrows).

Figure 3 | **Roles of the Argonaute complex in miRNA and RNAi pathways. a** | MicroRNA pathway. In animals, Argonaute associated with micro (mi)RNA binds to the 3' untranslated region (3'UTR) of mRNA and prevents the production of proteins in different ways. The recruitment of Argonaute proteins to targeted mRNA can induce deadenylation of the polyadenylated 3'end and induce mRNA degradation. The Argonaute–miRNA complex can also affect: the formation of functional ribosomes by abrogating the recruitment of ribosomal proteins to the 5' end of the mRNA and/or the production of growing polypeptides. **b** | RNAi pathway. Argonaute associated with small interfering (si)RNA forms the active RISC complex, which can induce endonucleolytic cleavage of targeted mRNA. In plants and *C. elegans* (boxed pathway), RNA-dependent RNA polymerases (RdRPs; yellow) contribute to the maintenance and propagation of the RNAi response throughout the organism. Once *de novo* dsRNA molecules are generated on the targeted mRNA, an unknown RNase III-like enzyme will produce new siRNAs called secondary siRNAs, which are then loaded onto a subclass of Argonaute (the secondary Argonaute), which, in turn, might induce another level of specific gene silencing. Alternatively, since a large population of secondary siRNAs isolated in *C. elegans* begins with the 5' di- or triphosphate group [64, 65], these small RNA species may also be produced by non-processives RdRPs found in nematodes (not shown).

Tables

Table 1 | **Functions of Argonaute proteins in different organisms.**

Organism	Molecular function	References
Neurospora crassa		
QDE2	Quelling	120, 121
SMS-2	Meiotic silencing of unpaired DNA	122
Schizosaccharomyces pombe		
Ago1	Heterochromatin silencing, TGS, PTGS	11, 12, 104, 123
Tetrahymena		
Twi1	DNA elimination	124
Arabidopsis thaliana		
AGO1	miRNA-mediated gene silencing, tasiRNA	125, 126
AGO4	rasiRNA, heterochromatin silencing	127
AGO6	rasiRNA, heterochromatin silencing	128
AGO7	tasiRNA, heteroblasty, leaf development	129
Caenorhabditis elegans		
RDE-1	Exogenous RNAi	7, 60
ALG-1	miRNA-mediated gene silencing, TGS	61, 130
ALG-2	miRNA-mediated gene silencing	61
ERGO-1	Endogenous RNAi	7
CSR-1	Chromosome segregation and RNAi	7
SAGO-1	Endogenous and exogenous RNAi	7
SAGO-2	Endogenous and exogenous RNAi	7
PPW-1	Endogenous and exogenous RNAi	7, 131
PPW-2	Endogenous and exogenous RNAi	7
F58G1.1	Endogenous and exogenous RNAi	7
C16C10.3	Endogenous and exogenous RNAi	7
PRG-1	Germline maintenance	91
Drosophila melanogaster		
Ago1	miRNA-mediated gene silencing	59
Ago2	RNAi	132
Ago3	piRNA, transposon silencing	37, 38
Piwi	piRNA, transposon silencing, germline stem-cell maintenance, RNAi	37, 38, 99
Aubergine	piRNA, transposon silencing, stellate silencing, DNA damage, RNAi	37, 38, 98
Zebrafish		
Ziwi	piRNA, germ-cell maintenance, transposon silencing	100
Murine/Human		
Ago1	Heterochromatin silencing	133, 134
Ago2	RNAi, miRNA-mediated gene silencing, heterochromatin silencing	39, 41, 133
Miwi (mouse)	piRNA, spermatogenesis	89, 93, 96
Mili (mouse)	piRNA, spermatogenesis	88, 97

Riwi (rat)	piRNA	[95]

The table contains Argonaute and Piwi proteins that have been associated with either a small RNA or cellular functions. Mammals encode two further Argonaute and Piwi proteins, *Arabidopsis thaliana* has six further Argonaute proteins and *C. elegans* has 15 further Argonaute proteins with no described function(s). Relevant references are listed.

miRNA, microRNA; piRNA, Piwi-interacting RNA; TGS, transcriptional gene silencing; PTGS, post-transcriptional gene silencing; rasiRNA, repeat-associated small interfering RNA; RNAi, RNA interference; tasiRNA, *trans*-acting small interfering RNA.

References

1. Moussian, B., Schoof, H., Haecker, A., Jurgens, G. & Laux, T. Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during Arabidopsis embryogenesis. *Embo J* **17**, 1799-1809 (1998).
2. Bohmert, K. et al. AGO1 defines a novel locus of Arabidopsis controlling leaf development. *Embo J* **17**, 170-180 (1998).
3. Lin, H. & Spradling, A.C. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. *Development* **124**, 2463-2476 (1997).
4. Dunoyer, P., Himber, C., Ruiz-Ferrer, V., Alioua, A. & Voinnet, O. Intra- and intercellular RNA interference in Arabidopsis thaliana requires components of the microRNA and heterochromatic silencing pathways. *Nat Genet* **39**, 848-856 (2007).
5. Zaratiegui, M., Irvine, D.V. & Martienssen, R.A. Noncoding RNAs and gene silencing. *Cell* **128**, 763-776 (2007).
6. Grewal, S.I. & Jia, S. Heterochromatin revisited. *Nat Rev Genet* **8**, 35-46 (2007).
7. Yigit, E. et al. Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. *Cell* **127**, 747-757 (2006).
8. Cerutti, H. & Casas-Mollano, J.A. On the origin and functions of RNA-mediated silencing: from protists to man. *Curr Genet* **50**, 81-99 (2006).
9. Ullu, E., Tschudi, C. & Chakraborty, T. RNA interference in protozoan parasites. *Cell Microbiol* **6**, 509-519 (2004).
10. Hall, I.M. et al. Establishment and maintenance of a heterochromatin domain. *Science* **297**, 2232-2237 (2002).
11. Volpe, T.A. et al. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833-1837 (2002).
12. Sigova, A., Rhind, N. & Zamore, P.D. A single Argonaute protein mediates both transcriptional and posttranscriptional silencing in Schizosaccharomyces pombe. *Genes Dev* **18**, 2359-2367 (2004).
13. Yuan, Y.R. et al. Crystal structure of A. aeolicus argonaute, a site-specific DNA-guided endoribonuclease, provides insights into RISC-mediated mRNA cleavage. *Mol Cell* **19**, 405-419 (2005).
14. Ma, J.B. et al. Structural basis for 5'-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. *Nature* **434**, 666-670 (2005).
15. Song, J.J., Smith, S.K., Hannon, G.J. & Joshua-Tor, L. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**, 1434-1437 (2004).
16. Song, J.J. et al. The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat Struct Biol* **10**, 1026-1032 (2003).
17. Yan, K.S. et al. Structure and conserved RNA binding of the PAZ domain. *Nature* **426**, 468-474 (2003).

18. Lingel, A., Simon, B., Izaurralde, E. & Sattler, M. Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. *Nature* **426**, 465-469 (2003).
19. Ma, J.B., Ye, K. & Patel, D.J. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature* **429**, 318-322 (2004).
20. Lingel, A., Simon, B., Izaurralde, E. & Sattler, M. Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. *Nat Struct Mol Biol* **11**, 576-577 (2004).
21. Parker, J.S., Roe, S.M. & Barford, D. Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *Embo J* **23**, 4727-4737 (2004).
22. Tolia, N.H. & Joshua-Tor, L. Slicer and the argonautes. *Nat Chem Biol* **3**, 36-43 (2007).
23. Martinez, J. & Tuschl, T. RISC is a 5' phosphomonoester-producing RNA endonuclease. *Genes Dev* **18**, 975-980 (2004).
24. Schwarz, D.S., Tomari, Y. & Zamore, P.D. The RNA-induced silencing complex is a Mg2+-dependent endonuclease. *Curr Biol* **14**, 787-791 (2004).
25. Parker, J.S., Roe, S.M. & Barford, D. Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. *Nature* **434**, 663-666 (2005).
26. Nykanen, A., Haley, B. & Zamore, P.D. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309-321 (2001).
27. Kiriakidou, M. et al. An mRNA m(7)G Cap Binding-like Motif within Human Ago2 Represses Translation. *Cell* **129**, 1141-1151 (2007).
28. Doench, J.G. & Sharp, P.A. Specificity of microRNA target selection in translational repression. *Genes Dev* **18**, 504-511 (2004).
29. Haley, B. & Zamore, P.D. Kinetic analysis of the RNAi enzyme complex. *Nat Struct Mol Biol* **11**, 599-606 (2004).
30. Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P. & Burge, C.B. Prediction of mammalian microRNA targets. *Cell* **115**, 787-798 (2003).
31. Elbashir, S.M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15**, 188-200 (2001).
32. Rivas, F.V. et al. Purified Argonaute2 and an siRNA form recombinant human RISC. *Nat Struct Mol Biol* **12**, 340-349 (2005).
33. Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H. & Siomi, M.C. Slicer function of Drosophila Argonautes and its involvement in RISC formation. *Genes Dev* **19**, 2837-2848 (2005).
34. Matranga, C., Tomari, Y., Shin, C., Bartel, D.P. & Zamore, P.D. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**, 607-620 (2005).
35. Leuschner, P.J., Ameres, S.L., Kueng, S. & Martinez, J. Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO Rep* **7**, 314-320 (2006).
36. Hutvágner, G. & Zamore, P.D. A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**, 2056-2060 (2002).
37. Gunawardane, L.S. et al. A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in Drosophila. *Science* **315**, 1587-1590 (2007).
38. Brennecke, J. et al. Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell* **128**, 1089-1103 (2007).
39. Meister, G. et al. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* **15**, 185-197 (2004).
40. Meister, G., Landthaler, M., Dorsett, Y. & Tuschl, T. Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *Rna* **10**, 544-550 (2004).

41. Liu, J. et al. Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437-1441 (2004).
42. Hock, J. et al. Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. *EMBO Rep* (2007).
43. Tahbaz, N. et al. Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. *EMBO Rep* **5**, 189-194 (2004).
44. Behm-Ansmant, I. et al. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev* **20**, 1885-1898 (2006).
45. Till, S. et al. A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat Struct Mol Biol* **14**, 897-903 (2007).
46. El-Shami, M. et al. Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. *Genes Dev* **21**, 2539-2544 (2007).
47. Schwarz, D.S. et al. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199-208 (2003).
48. Khvorova, A., Reynolds, A. & Jayasena, S.D. Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**, 209-216 (2003).
49. Forstemann, K. et al. Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol* **3**, e236 (2005).
50. Saito, K., Ishizuka, A., Siomi, H. & Siomi, M.C. Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells. *PLoS Biol* **3**, e235 (2005).
51. Jiang, F. et al. Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila. *Genes Dev* **19**, 1674-1679 (2005).
52. Liu, Q. et al. R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science* **301**, 1921-1925 (2003).
53. Tomari, Y., Matranga, C., Haley, B., Martinez, N. & Zamore, P.D. A protein sensor for siRNA asymmetry. *Science* **306**, 1377-1380 (2004).
54. Forstemann, K., Horwich, M.D., Wee, L., Tomari, Y. & Zamore, P.D. Drosophila microRNAs Are Sorted into Functionally Distinct Argonaute Complexes after Production by Dicer-1. *Cell* **130**, 287-297 (2007).
55. Tomari, Y., Du, T. & Zamore, P.D. Sorting of Drosophila Small Silencing RNAs. *Cell* **130**, 299-308 (2007).
56. Steiner, F.A. et al. Structural features of small RNA precursors determine Argonaute loading in Caenorhabditis elegans. *Nat Struct Mol Biol* (2007).
57. Tomari, Y. et al. RISC assembly defects in the Drosophila RNAi mutant armitage. *Cell* **116**, 831-841 (2004).
58. Tomari, Y. & Zamore, P.D. Perspective: machines for RNAi. *Genes Dev* **19**, 517-529 (2005).
59. Okamura, K., Ishizuka, A., Siomi, H. & Siomi, M.C. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev* **18**, 1655-1666 (2004).
60. Tabara, H. et al. The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell* **99**, 123-132 (1999).
61. Grishok, A. et al. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* **106**, 23-34 (2001).

62. Reinhart, B.J. et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403**, 901-906 (2000).
63. Lee, R.C., Feinbaum, R.L. & Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843-854 (1993).
64. Sijen, T., Steiner, F.A., Thijssen, K.L. & Plasterk, R.H. Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science* **315**, 244-247 (2007).
65. Pak, J. & Fire, A. Distinct populations of primary and secondary effectors during RNAi in C. elegans. *Science* **315**, 241-244 (2007).
66. Vasudevan, S. & Steitz, J.A. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. *Cell* **128**, 1105-1118 (2007).
67. Olsen, P.H. & Ambros, V. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. *Dev Biol* **216**, 671-680 (1999).
68. Wang, B., Love, T.M., Call, M.E., Doench, J.G. & Novina, C.D. Recapitulation of short RNA-directed translational gene silencing in vitro. *Mol Cell* **22**, 553-560 (2006).
69. Humphreys, D.T., Westman, B.J., Martin, D.I. & Preiss, T. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc Natl Acad Sci U S A* **102**, 16961-16966 (2005).
70. Pillai, R.S. et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309**, 1573-1576 (2005).
71. Thermann, R. & Hentze, M.W. Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. *Nature* **447**, 875-878 (2007).
72. Chendrimada, T.P. et al. MicroRNA silencing through RISC recruitment of eIF6. *Nature* **447**, 823-828 (2007).
73. Kim, J. et al. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. *Proc Natl Acad Sci U S A* **101**, 360-365 (2004).
74. Nelson, P.T., Hatzigeorgiou, A.G. & Mourelatos, Z. miRNP:mRNA association in polyribosomes in a human neuronal cell line. *Rna* **10**, 387-394 (2004).
75. Nottrott, S., Simard, M.J. & Richter, J.D. Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol* **13**, 1108-1114 (2006).
76. Maroney, P.A., Yu, Y., Fisher, J. & Nilsen, T.W. Evidence that microRNAs are associated with translating messenger RNAs in human cells. *Nat Struct Mol Biol* **13**, 1102-1107 (2006).
77. Petersen, C.P., Bordeleau, M.E., Pelletier, J. & Sharp, P.A. Short RNAs repress translation after initiation in mammalian cells. *Mol Cell* **21**, 533-542 (2006).
78. Pillai, R.S., Artus, C.G. & Filipowicz, W. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *Rna* **10**, 1518-1525 (2004).
79. Bhattacharyya, S.N., Habermacher, R., Martine, U., Closs, E.I. & Filipowicz, W. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* **125**, 1111-1124 (2006).
80. Rehwinkel, J. et al. Genome-wide analysis of mRNAs regulated by Drosha and Argonaute proteins in Drosophila melanogaster. *Mol Cell Biol* **26**, 2965-2975 (2006).
81. Giraldez, A.J. et al. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**, 75-79 (2006).
82. Lim, L.P. et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433**, 769-773 (2005).
83. Farh, K.K. et al. The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. *Science* **310**, 1817-1821 (2005).

84. Bagga, S. et al. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* **122**, 553-563 (2005).
85. Wu, L., Fan, J. & Belasco, J.G. MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A* **103**, 4034-4039 (2006).
86. Valencia-Sanchez, M.A., Liu, J., Hannon, G.J. & Parker, R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* **20**, 515-524 (2006).
87. Jing, Q. et al. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* **120**, 623-634 (2005).
88. Kuramochi-Miyagawa, S. et al. Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. *Development* **131**, 839-849 (2004).
89. Deng, W. & Lin, H. miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. *Dev Cell* **2**, 819-830 (2002).
90. Cox, D.N., Chao, A. & Lin, H. piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503-514 (2000).
91. Cox, D.N. et al. A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev* **12**, 3715-3727 (1998).
92. Carmell, M.A. et al. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Dev Cell* **12**, 503-514 (2007).
93. Grivna, S.T., Beyret, E., Wang, Z. & Lin, H. A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev* **20**, 1709-1714 (2006).
94. Watanabe, T. et al. Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. *Genes Dev* **20**, 1732-1743 (2006).
95. Lau, N.C. et al. Characterization of the piRNA complex from rat testes. *Science* **313**, 363-367 (2006).
96. Girard, A., Sachidanandam, R., Hannon, G.J. & Carmell, M.A. A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature* **442**, 199-202 (2006).
97. Aravin, A. et al. A novel class of small RNAs bind to MILI protein in mouse testes. *Nature* **442**, 203-207 (2006).
98. Vagin, V.V. et al. A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**, 320-324 (2006).
99. Saito, K. et al. Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. *Genes Dev* **20**, 2214-2222 (2006).
100. Houwing, S. et al. A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. *Cell* **129**, 69-82 (2007).
101. Pane, A., Wehr, K. & Schupbach, T. zucchini and squash encode two putative nucleases required for rasiRNA production in the Drosophila germline. *Dev Cell* **12**, 851-862 (2007).
102. Brower-Toland, B. et al. Drosophila PIWI associates with chromatin and interacts directly with HP1a. *Genes Dev* **21**, 2300-2311 (2007).
103. Hiragami, K. & Festenstein, R. Heterochromatin protein 1: a pervasive controlling influence. *Cell Mol Life Sci* **62**, 2711-2726 (2005).
104. Noma, K. et al. RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* **36**, 1174-1180 (2004).
105. Yin, H. & Lin, H. An epigenetic activation role of Piwi and a Piwi-associated piRNA in Drosophila melanogaster. *Nature* (2007).

106. Cerutti, L., Mian, N. & Bateman, A. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem Sci* **25**, 481-482 (2000).
107. Jacobsen, S.E., Running, M.P. & Meyerowitz, E.M. Disruption of an RNA helicase/RNAse III gene in Arabidopsis causes unregulated cell division in floral meristems. *Development* **126**, 5231-5243 (1999).
108. Kirino, Y. & Mourelatos, Z. Mouse Piwi-interacting RNAs are 2'-O-methylated at their 3' termini. *Nat Struct Mol Biol* **14**, 347-348 (2007).
109. Saito, K. et al. Pimet, the Drosophila homolog of HEN1, mediates 2'-O-methylation of Piwi-interacting RNAs at their 3' ends. *Genes Dev* **21**, 1603-1608 (2007).
110. Horwich, M.D. et al. The Drosophila RNA Methyltransferase, DmHen1, Modifies Germline piRNAs and Single-Stranded siRNAs in RISC. *Curr Biol* **17**, 1265-1272 (2007).
111. Lee, Y. et al. MicroRNA genes are transcribed by RNA polymerase II. *Embo J* **23**, 4051-4060 (2004).
112. Borchert, G.M., Lanier, W. & Davidson, B.L. RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* **13**, 1097-1101 (2006).
113. Lee, Y. et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-419 (2003).
114. Okamura, K., Hagen, J.W., Duan, H., Tyler, D.M. & Lai, E.C. The Mirtron Pathway Generates microRNA-Class Regulatory RNAs in Drosophila. *Cell* **130**, 89-100 (2007).
115. Ruby, J.G., Jan, C.H. & Bartel, D.P. Intronic microRNA precursors that bypass Drosha processing. *Nature* **448**, 83-86 (2007).
116. Yi, R., Qin, Y., Macara, I.G. & Cullen, B.R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**, 3011-3016 (2003).
117. Ketting, R.F. et al. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev* **15**, 2654-2659 (2001).
118. Hutvágner, G. et al. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293**, 834-838 (2001).
119. Mathews, D.H., Sabina, J., Zuker, M. & Turner, D.H. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* **288**, 911-940 (1999).
120. Cogoni, C. & Macino, G. Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in Neurospora crassa. *Proc Natl Acad Sci U S A* **94**, 10233-10238 (1997).
121. Catalanotto, C., Azzalin, G., Macino, G. & Cogoni, C. Gene silencing in worms and fungi. *Nature* **404**, 245 (2000).
122. Lee, D.W., Pratt, R.J., McLaughlin, M. & Aramayo, R. An argonaute-like protein is required for meiotic silencing. *Genetics* **164**, 821-828 (2003).
123. Verdel, A. et al. RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**, 672-676 (2004).
124. Mochizuki, K., Fine, N.A., Fujisawa, T. & Gorovsky, M.A. Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in tetrahymena. *Cell* **110**, 689-699 (2002).
125. Vazquez, F. et al. Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. *Mol Cell* **16**, 69-79 (2004).
126. Vaucheret, H., Vazquez, F., Crete, P. & Bartel, D.P. The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* **18**, 1187-1197 (2004).

127. Zilberman, D., Cao, X. & Jacobsen, S.E. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299**, 716-719 (2003).
128. Zheng, X., Zhu, J., Kapoor, A. & Zhu, J.K. Role of Arabidopsis AGO6 in siRNA accumulation, DNA methylation and transcriptional gene silencing. *Embo J* **26**, 1691-1701 (2007).
129. Hunter, C., Sun, H. & Poethig, R.S. The Arabidopsis heterochronic gene ZIPPY is an ARGONAUTE family member. *Curr Biol* **13**, 1734-1739 (2003).
130. Grishok, A., Sinskey, J.L. & Sharp, P.A. Transcriptional silencing of a transgene by RNAi in the soma of C. elegans. *Genes Dev* **19**, 683-696 (2005).
131. Tijsterman, M., Okihara, K.L., Thijssen, K. & Plasterk, R.H. PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of C. elegans. *Curr Biol* **12**, 1535-1540 (2002).
132. Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R. & Hannon, G.J. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**, 1146-1150 (2001).
133. Janowski, B.A. et al. Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. *Nat Struct Mol Biol* **13**, 787-792 (2006).
134. Kim, D.H., Villeneuve, L.M., Morris, K.V. & Rossi, J.J. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat Struct Mol Biol* **13**, 793-797 (2006).

Glossary terms

RNA induced silencing complex (RISC)

A multicomponent gene regulatory complex, activated by a small RNA associated with Argonaute or Piwi proteins, that cleaves specific mRNAs that are targeted for degradation by homologous dsRNAs during the process of RNA interference.

Exogenous RNAi

A silencing response mediated by exogenous experimentally delivered double-stranded RNA molecules.

Endogenous RNAi

An RNAi response initiated by endogenous double-stranded RNA triggers derived from bi-directional transcription of specific loci, or aberrant RNA generated from centromeric regions, transposons and transgenes.

RasiRNA

Repeat-associated small interfering RNA derived from highly repetitive genomic loci. RasiRNA is involved in heterochromatin silencing in yeast and plants and stellate silencing in *D. melanogaster*. Metazoan rasiRNAs have similarities with piRNAs, as both classes of processing are independent of Dicer and Drosha.

TasiRNA

Trans-acting small interfering RNAs are plant-specific small RNAs and their maturation involves miRNAs. miRNAs cleave the single-stranded primary transcript, which is further amplified by RNA-dependent RNA polymerases followed by Dicer-mediated processing of *de novo* dsRNA molecules. The generated siRNAs are then incorporated into Argonaute complexes and regulate gene expression by cleaving the target RNA.

RNase H

A class of RNA endonucleases that cleaves the RNA strand of a DNA–RNA duplex. Argonaute and Piwi proteins share similar catalytic domain structure and activity with RNase H enzymes but are mostly active on RNA–RNA hybrids.

RNAi pathway.

The pathway by which expression or transfection of double-stranded RNA induces degradation — by nucleases — of the homologous endogenous transcripts. This mimics the effect of the reduction, or loss, of gene activity.

microRNA (miRNA)

A non-coding RNA of 21–24 nucleotides, which is processed from an endogenous ~70-nucleotide hairpin RNA precursor by the RNase-III-type Dicer enzyme. miRNAs are evolutionarily conserved molecules and are thought to have important functions in various biological mechanisms.

Small Interfering RNA (siRNA)

A short RNA (~22 nucleotides) that is processed from longer dsRNA during RNAi. These short RNAs hybridize with mRNA targets, and confer target specificity to the silencing complexes in which they reside.

PAZ domain

A conserved nucleic-acid-binding structure that is found in members of the Dicer and Argonaute protein families.

PIWI domain

A conserved structure that is found in members of the Argonaute protein family. It is structurally similar to ribonuclease-H domains and, in at least some cases, has endoribonuclease activity.

PIWI-box

A 40-amino-acid sequence located in the C terminus of the Piwi-like protein.

CAP structure

A structure, which consists of m^7GpppN (where m^7G represents 7-methylguanylate, p represents a phosphate group and N represents any base), that is located at the 5' end of eukaryotic mRNAs.

Paralogous, **Orthologous**

The quality of having sequence similarity as a result of gene duplication events that occurred in the same genome (paralogous) or in a different genome (orthologous).

Poly(A) tail

A homopolymeric stretch of usually 25–200 adenine nucleotides that is present at the 3' end of most eukaryotic mRNAs.

Mobile elements

Also known as transposable elements. DNA sequences in the genome that replicate and insert themselves into various positions in the genome.

Deadenylation-dependent decapping

Cytoplasmic RNA degradation that start with the depletion of the poly(A) tail of a mRNA followed by removing the cap by decapping enzymes. The decapped RNA is degraded by 5´-3´exonucleases.

Heterochronic phenotypes

Animals that display observable characteristics related to a specific defect in the developmental timing (i.e. larvae that displays adult characteristics or adult animal with larval features).

Active strand

The strand of a duplex siRNA or miRNA intermediate that dominantly selected and incorporated into the RISC.

Passenger strand

The strand of a duplex siRNA or miRNA that not incorporated into RISC and eventually degraded.

Piwi-interacting (pi)RNAs

Small ~31nt long RNAs that processed a Dicer and Drosha independent manner. They associated with Piwi proteins and have role in transposon silencing in flies. In mammals they are restricted mostly in the male germ cells.

Cytoplasmic processing bodies

Cytoplasmic foci that was first detected by the immunostaining with GW182 Antibody. This foci is very likely a protein RNA aggregate that degrade RNAs via deadenylation and decapping. It also accommodate Argonaute bound miRNAs and miRNA targeted RNAs. Cytoplasmic bodies does not form without miRNAs however disruption the cytoplasmic body does not affect miRNA mediated gene regulation.

OB (oligonucleotide/oligosaccharide binding)-fold

Common protein structure involved in nucleic acid binding.

miRNA pathway

The process that result in a functional miRNA loaded RNA-protein complex. It includes miRNA expression, maturation, miRNA loading, and miRNA-loaded RISC formation (also called miRISC).

Dicer

The ribonuclease of the RNAse III family that cleaves miRNA precursor (pre-miRNA) and double-stranded RNA molecules into 21-25 long double-stranded RNA with a two-base overhang on the 3’ ends.

Drosha

The RNAse III enzyme implicated in the processing into the nucleus of the newly transcribed primary miRNA. The Drosha cleavage will determine the 5’ and the 3’ ends of the Dicer substrate (precursor miRNA or pre-miRNA).