NON-CODING RNA

Argonaute proteins: functional insights and emerging roles

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Abstract | Small-RNA-guided gene regulation has emerged as one of the fundamental principles in cell function, and the major protein players in this process are members of the Argonaute protein family. Argonaute proteins are highly specialized binding modules that accommodate the small RNA component — such as microRNAs (miRNAs), short interfering RNAs (siRNAs) or PIWI-associated RNAs (piRNAs) — and coordinate downstream gene-silencing events by interacting with other protein factors. Recent work has made progress in our understanding of classical Argonaute-mediated gene-silencing principles, such as the effects on mRNA translation and decay, but has also implicated Argonaute proteins in several other cellular processes, such as transcriptional regulation and splicing.

Argonaute proteins are key players in all small-RNA-guided gene-silencing processes that have been identified and characterized so far. They are highly conserved, and family members are found in all eukaryotes, with the exception of *Saccharomyces cerevisiae*, which has lost the small RNA machinery[1]. Also, some bacteria and archaea contain Argonaute genes[2]: their specific functions, however, remain elusive.

Argonaute proteins are the direct binding partners of small RNAs. The eukaryotic Argonaute family can be divided into AGO proteins (also referred to as the AGO clade), which are similar to *Arabidopsis thaliana* AGO1, and PIWI proteins (also referred to as the PIWI clade), which are homologous to *Drosophila melanogaster* Piwi. AGO proteins mainly interact with microRNAs (miRNAs) or short interfering RNAs (siRNAs) and are involved in cytoplasmic post-transcriptional gene-silencing processes[3,4]. PIWI proteins are mainly expressed in germline cells, where these proteins bind to PIWI-interacting RNAs (piRNAs) and function in the silencing of transposable genetic elements[5]. In *Caenorhabditis elegans*, in which 26 different Argonaute genes exist, a third clade (known as WAGO) has evolved that is equally distant from the AGO and PIWI clades. These proteins mainly serve as secondary Argonaute proteins and acquire their small RNA load through 'primary' Argonaute proteins or through small RNA amplification processes[6]. Plant Argonaute proteins cluster to the AGO clade[2]. However, some of these proteins are exclusively expressed in the germ line and have plant-specific functions[7]. The biogenesis of small RNA classes is summarized in BOXES 1,2.

In the past few years, novel insights into the function of Argonaute proteins have been reported. Among them are additional mechanistic details that improve our understanding of known aspects, such as structural information on human Argonaute proteins, the mechanism of small RNA loading, Argonaute post-translational modifications and Argonaute protein function in germ cells. In addition, novel cellular Argonaute functions are emerging, including roles for Argonaute in transcription, alternative splicing and even DNA repair. This Review focuses on the recent literature of the diverse aspects of Argonaute protein function with an emphasis on AGO clade proteins. The first part discusses insights into known functions, and the second part addresses novel pathways in which Argonaute proteins have been implicated.

Structural insights

Structural studies have revealed how Argonaute proteins act as highly specialized small-RNA-binding modules. They are characterized by amino-terminal (N), PAZ (PIWI–ARGONAUTE–ZWILLE), MID (middle) and PIWI domains. Initial structural studies on bacterial and archaeal Argonaute proteins revealed that Argonaute proteins consist of two lobes comprising the N–PAZ and the MID–PIWI domains, respectively. A hinge connects the two lobes, and structural rearrangements occur during RNA binding. The PAZ domain anchors the 3′ end of the small RNA by bending it into a specific binding pocket[8]. PAZ domains of the PIWI subfamily members specifically accommodate methylated piRNA 3′ ends[9]. The MID domain

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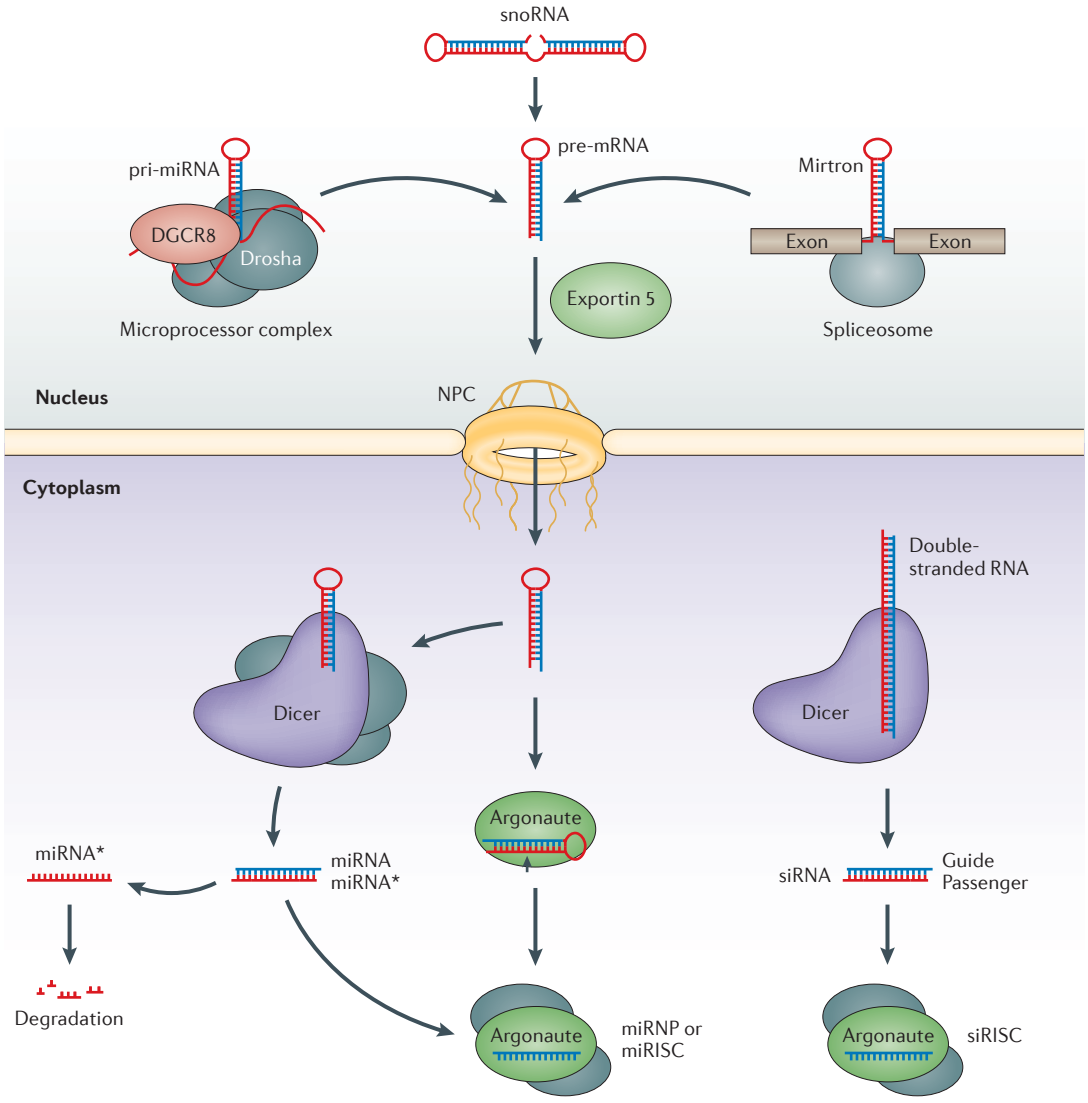
Box 1 | **Biogenesis of miRNAs and siRNAs**



Most microRNAs (miRNAs) are transcribed as primary transcripts (pri-miRNAs) by RNA polymerase II (RNA Pol II; see the upper left panel of the figure). Such transcripts contain one or more hairpins, and the miRNAs lie in their double-stranded stem. Processing by the nuclear microprocessor complex, which contains the RNase III enzyme Drosha, releases the hairpin: this part is referred to as the precursor miRNA (pre-miRNA)[70]. Pre-miRNAs can also be generated from short introns, which are referred to as mirtrons[154] (see the upper right panel of the figure). Mirtrons are Drosha-independent and are liberated during splicing by the spliceosome. Some classes of small nucleolar RNAs (snoRNAs) can also produce miRNAs[155] (see the upper centre panel of the figure); how these are processed is unclear. After export to the cytoplasm by exportin 5, Dicer (another RNase III enzyme) cleaves off the loop of the hairpin and generates a double-stranded RNA. The mature miRNA strand is subsequently incorporated into the RNA-induced silencing complex (RISC, or miRISC for miRNA-containing RISC or miRNP for microribonucleoprotein), where it directly binds to a member of the AGO protein family. The other strand is referred to as miRNA* and is normally degraded. In some cases, miRNAs* can be functional as well[70]. It has also been shown that miRNAs can be processed independently of Dicer. AGO2, for example, can cleave miRNA precursors and produce mature miRNAs[67,156–159].

Small interfering RNAs (siRNAs) are very similar to miRNAs and are widely used as research tools for sequence-specific gene inactivation. Depending on the organism, siRNAs are either introduced as short double-stranded RNAs (dsRNAs) by transfection or long dsRNAs, which are processed to siRNAs by Dicer[73] (see the lower right panel of the figure). The so-called guide strand is incorporated into RISC (or siRISC for siRNA-containing RISC), and the other strand (known as the passenger strand) is destabilized. In many organisms, siRNAs from endogenous transcripts have been identified. In *Drosophila melanogaster* gonadal and somatic tissues, as well as cultured S2 cells, endogenous siRNAs mainly correspond to a subset of retrotransposons or stem–loop-structured RNAs[160–162]. In mouse oocytes, endogenous siRNAs derive from different dsRNA sources, including inverted repeat structures, bidirectional transcription or antisense transcription[163,164]. Pseudogenes seem to be sources for endogenous siRNAs as well. It is currently unclear, however, whether such endogenous siRNAs exist in mammalian somatic cells as well. NPC, nuclear pore complex.

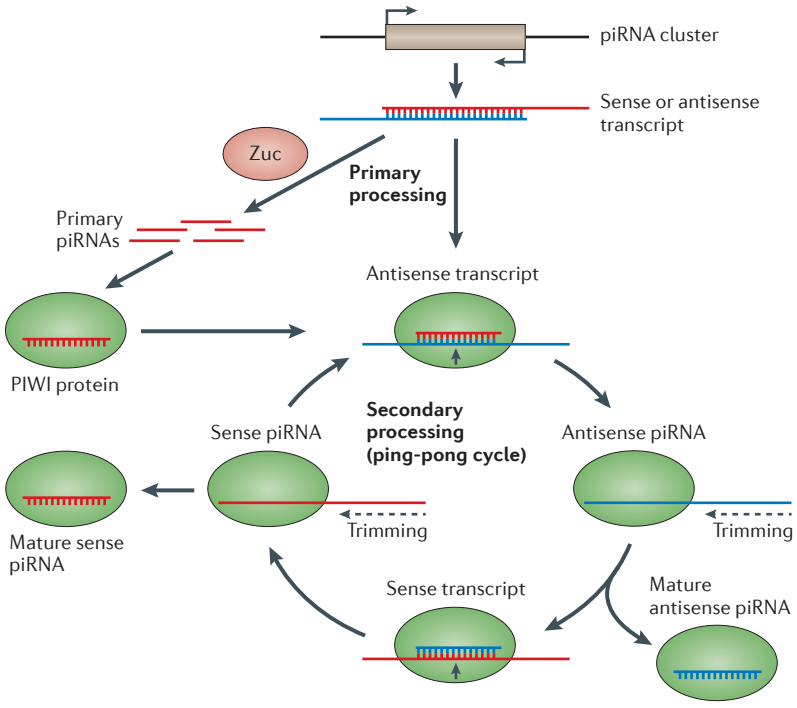
Box 2 | **piRNA biogenesis and function**

Mobile genetic elements such as transposons are a constant threat for the genome. PIWI-interacting RNAs (piRNAs) protect germline cells from transposons in organisms as diverse as flies, fish or mammals. piRNAs are 25 to 33 nt in length, depending on the PIWI clade protein that they bind to. piRNAs derive from distinct transposons that are referred to as piRNA clusters, but the piRNAs from each locus are characterized by a complex mixture of sequences spanning large portions of the transposon[5]. piRNA clusters are transcribed in the sense or antisense direction, and the long single-stranded RNA serves as the basis for piRNA production.

The biogenesis of piRNAs is independent of Dicer[165] and requires other nucleases. Two biogenesis pathways are important for piRNA production. First, a primary processing pathway generates primary piRNAs, and these are then amplified by an amplification cycle referred to as the ping-pong loop[5]. In the primary biogenesis pathway, the long transposon transcript is initially cleaved by the nuclease zucchini (Zuc; see the figure)[166–168], which probably generates the 5′ ends of primary piRNAs. The other steps of primary piRNA maturation are not understood[5].

In the ping-pong cycle (see the lower right panel of the figure), mature sense primary piRNAs guide PIWI clade proteins to complementary sequences on antisense transcripts from the same piRNA cluster. PIWI proteins use their slicer activity to cleave the target antisense transcript to generate a new 5′ end. This 5′ end is bound by another PIWI protein. In subsequent steps, the 3′ end is trimmed to the length of the mature piRNA, leading to a mature antisense secondary piRNA, which can now target sense transcripts transcribed from the piRNA cluster. In *Drosophila melanogaster*, the two Piwi proteins Aubergine and Ago3 cooperate in secondary piRNA production to generate sense and antisense piRNAs. However, antisense piRNAs dominate, and a protein called Qin, which contains E3 ligase and Tudor domains, seems to modulate such a heterotypic ping-pong cycle[169]. In the mouse germ line, the PIWI proteins MILI and MIWI collaborate in piRNA generation. After trimming, piRNAs receive a methyl group at the 3′ end by the methyltransferase HEN1. Primary piRNAs carry such modifications as well[5]. In *Caenorhabditis elegans*, an additional piRNA production pathway exists. Short and capped RNA polymerase II transcripts are decapped, processed and directly loaded into PIWI proteins[170].

piRNAs guide PIWI proteins to complementary RNAs derived from transposable elements. Similarly to in RNA interference, PIWI proteins cleave the transposon RNA, leading to silencing. In flies, mutations in *piwi*, *aub* and *Ago3* (which encode the Piwi proteins in *D. melanogaster*) are required for transposon silencing in the germ line[5]. Similar observations were made when the mouse PIWI proteins MILI and MIWI were genetically inactivated[171]. Here, long interspersed nuclear elements (LINE) and long terminal repeat (LTR) retrotransposons accumulated[172].



anchors the 5′ end of the small RNA by providing a binding pocket in which the 5′ terminal base engages in stacking interactions with a conserved tyrosine. In addition, several hydrogen bonds coordinate correct 5′ end binding[8]. The N domain is required for small RNA loading and assists in unwinding the small RNA duplex[10].

The PIWI domain is structurally similar to RNase H, and it has indeed been shown that Argonaute proteins can function as endonucleases and cleave target RNA that is fully complementary to the bound small RNA[8]. The PIWI domain contains a catalytic triad composed of DDX (where X is D or H). Recent structural work on an Argonaute protein from yeast revealed that a fourth residue is also essential turning the catalytic centre into a tetrad of DEDX[11]. Generally, only a subset of Argonaute proteins possesses cleavage activity. In mammals, for example, only AGO2 of the AGO subfamily is catalytically active and functions as an endonuclease[12,13]. Besides the PIWI domain, an unstructured loop in the N domain is also important for cleavage[14].

Recently, crystal structures of human AGO2 have been reported[15,16]. Overall, their structure is strikingly similar to known bacterial and archaeal Argonaute proteins, underlining the high conservation of the basic principles of small RNA pathways.

Mechanisms of Argonaute loading

The AGO subfamily and the PIWI subfamily use different mechanisms for small RNA loading, and they will be discussed separately with an emphasis on AGO clade proteins.

Loading AGO clade members. Dicer processing generates a short double-stranded RNA of about 20–24 nt in length. However, only one strand associates with the AGO protein and becomes the guide strand. How is the correct strand selected, and how is it loaded into AGO proteins? One important determinant for strand selection lies in the small RNA duplex itself: both in the miRNA pathway and the siRNA pathway, the strand with the less stably paired 5′ end is preferentially loaded into AGO proteins. These thermodynamic differences between the small RNA ends are known as the asymmetry rule[17,18].

In various organisms, Dicer proteins directly interact with AGO proteins and strand selection, and loading is achieved within such complexes[19–21]. In addition, Dicer proteins partner with various different dsRNA-binding proteins, which have been most extensively studied in *D. melanogaster*. In flies, two Dicer proteins exist: Dcr1 processes precursor miRNAs (pre-miRNAs) and loads Ago1 for miRNA-guided gene silencing, whereas Dcr2 cleaves perfectly paired long dsRNA and loads siRNAs into Ago2. Dcr1 interacts with loquacious (Loqs), which is essential for the miRNA pathway[22]. Dcr2 requires R2d2, which is important for the siRNA pathway. R2d2 functions as a sensor for the thermodynamic asymmetry within an siRNA duplex and positions Dcr2 for correct strand selection[23,24]. In mammals, the dsRNA-binding domain proteins TRBP

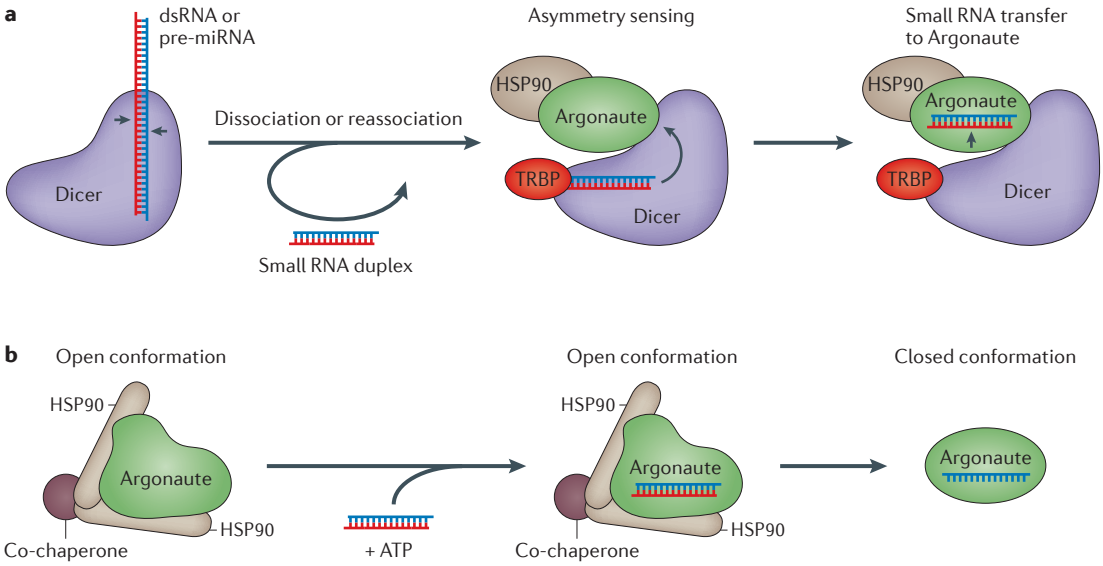


Figure 1 | **A model for loading of small RNAs into AGO clade proteins. a** | Dicer binds long double-stranded RNAs (dsRNAs) or precursor microRNAs (pre-miRNAs) and uses its two RNase III domains to cleave both strands (indicated by the two arrows), generating a ~21 nt dsRNA. This short dsRNA dissociates from and reassociates with Dicer at a different position. During this step, a dsRNA-binding protein (here, TRBP) senses the asymmetry and positions the RNA in an orientation, allowing correct strand selection and loading. In the next step, the dsRNA is transferred to a bound Argonaute protein that is kept in an open conformation by heat shock protein 90 (HSP90). Cleavage-competent AGO proteins cleave the passenger strand (indicated by an arrow), allowing for rapid strand displacement. Cleavage-incompetent AGO proteins displace the passenger strand through a much slower process without prior cleavage. See REF. 28 for further details. **b** | An HSP90 dimer binds unloaded Argonaute proteins and holds them in an open conformation. Co-chaperones support HSP90 in this process. After small RNA binding, the passenger strand is removed, HSP90 hydrolyses ATP, and the AGO protein loaded with the single-stranded RNA transitions into a closed conformation. HSP90 and potential co-chaperones leave the complex.

(also known as TARBP2) and PACT (also known as PRKRA) interact with Dicer and have been implicated in miRNA biogenesis[25–27].

Novel insights into how loading events might function in humans have come from recent biochemical studies[28]. First, it was confirmed that human TRBP is an asymmetry sensor and functions similarly to R2d2 in *D. melanogaster*. Furthermore, Dicer contains two different RNA-binding sites: one that positions long dsRNA for cleavage, and one that rebinds the cleaved siRNA after cleavage with the help of TRBP. During this second step, asymmetry sensing occurs (FIG. 1a). Within a minimal RNA-induced silencing complex-loading complex (RISC-loading complex) containing Dicer, TRBP and an AGO protein, the double-stranded siRNA or miRNA is loaded onto AGO. The transfer of the double-stranded siRNA or miRNA to the Ago protein is mediated by the multi-protein chaperone complex Hsc70–Hsp90 (heat shock cognate protein 70 kDa–heat shock protein 90) in *D. melanogaster* or HSP90 in plants and humans; this complex hydrolyses ATP to keep AGO proteins in an open state, allowing them to accommodate the RNA duplex[29–31] (FIG. 1b). Heat shock proteins are often assisted by co-chaperones. Indeed, a recent mass spectrometry study found that the co-chaperone FKBP5 associated with mouse AGO2 (REF. 32). Whether it is required for RISC loading remains to be shown.

In the next step, the duplex is unwound, and the passenger strand of the siRNA or the miRNA* strand of the miRNA is removed. There is recent evidence that the N domain of AGO proteins supports this step[10]. 'Wedging' of the N domain, in which there are conformational changes in the AGO protein that drive the N domain between the two duplex strands, leads to duplex opening and further unwinding. How unwinding occurs, and whether additional factors are needed, remains unclear. It has also been reported that AGO proteins themselves might possess strand dissociation activity to remove one of the strands[33]. Putative helicases, such as RNA helicase A (RHA), MOV10 or Armitage, have also been implicated in duplex unwinding in human and *D. melanogaster* cells[19,34–36]. Strand removal correlates with the overall thermodynamic stability of the duplex, possibly arguing against the action of additional helicases, which might act independently of sequence[35,37]. Further studies are required to solve this controversy.

Perfectly paired siRNA or miRNA duplexes are cleaved by catalytically active AGO proteins, such as AGO2 (REFS 38–40); this leads to efficient loading of catalytically active AGO proteins. AGO proteins that lack catalytic activity are loaded as well but require more time to remove the passenger strand. In flies and humans, the passenger strand nicked by AGO2 is removed from the RISC-loading complex by the endonuclease C3PO (also known as translin). C3PO interacts

Dicer
An RNase III family endonuclease that processes double-stranded RNA and precursor microRNAs into small interfering RNAs and microRNAs, respectively.

RNA-induced silencing complex-loading complex
(RISC-loading complex). A protein complex containing Dicer, a double-stranded RNA-binding protein, Argonaute, heat shock protein 90 (HSP90) and potentially other proteins that is required for loading small RNAs onto the Argonaute protein.

miRNA* strand
The microRNA (miRNA) strand within the miRNA precursor that is not selected as the mature miRNA. miRNA* strands are typically degraded and are therefore less abundant in cells.

with AGO2 and activates RISC, which is now ready to fully cleave complementary target RNAs[41,42]. It remains unknown whether C3PO also functions in conjunction with non-catalytic AGO proteins. A minimal RISC-loading complex requires Dicer, a dsRNA-binding protein, the HSC70–HSP90 system and an AGO protein. However, it is likely that additional species-specific or even sequence-specific factors influence RISC loading.

Loading PIWI clade members. Loading PIWI proteins with piRNAs is tightly linked to piRNA biogenesis, which is summarized in BOX 2. Although the piRNA-loading mechanisms are largely unknown, several recent reports shed some light on this process. In *D. melanogaster*, genetic studies found that loss of the putative helicase Armitage reduced the levels of PIWI, suggesting that is has a role in PIWI–RISC formation or stabilization[43]. Interestingly, the Armitage orthologue in mice, MOV10L1, is also required for piRNA production[44,45]. The detailed mechanisms, however, remain unclear. Recent genetic data in *D. melanogaster* revealed that all piRNA classes are lost when the gene encoding the co-chaperone shutdown (Shu) is mutated[46]; Shu interacts with Hsp90 (REFS 46,47). In mice, the co-chaperone FKBP6 seems to be required for the delivery of piRNAs to the PIWI protein MIWI2 (REF. 48). Therefore, it is likely that piRNA loading uses similar chaperone-assisted mechanisms to miRNA and siRNA loading in somatic cells.

Distinct AGO clade proteins

Biochemical isolation of Argonaute proteins followed by deep sequencing of the associated small RNAs has revealed that different classes of small RNAs can associate with distinct Argonaute proteins. In this section, examples for such associations in various organisms are discussed.

Drosophila melanogaster. Sorting small RNAs into various Ago clade proteins has been extensively studied in *D. melanogaster*. In flies, precursor RNAs with a near-perfect complementarity in the dsRNA are processed by Dcr2 and are specifically loaded into Ago2. By contrast, miRNA precursors, which are usually not fully paired, are processed by Dcr1 and loaded into Ago1 (REFS 49–52). However, there are also exceptions to these rules: miR-277, for example, is processed by Dcr1 but is loaded into Ago2. This process seems to involve rebinding to Dcr2, which loads miR-277 into Ago2 (REF. 50). In addition, endogenous siRNAs (endo-siRNAs), which derive from mismatched precursors, are found in Ago2. These findings highlight that there are additional mechanisms that influence loading of small RNAs in *D. melanogaster*[53,54].

Arabidopsis thaliana. In *A. thaliana*, ten different AGO proteins exist, and they also show preferences for distinct classes of small RNAs. In addition, four different Dicer variants, DCL1–4, are required for the production and loading of AGO proteins. For example, DCL1 generates 18–21 nt-long miRNAs and loads them into AGO1 (REF. 55). DCL2–4 produces slightly longer small RNAs, which are loaded into other Argonaute proteins. The mechanistic details, however, remain elusive[55]. AGO4 contains a specific class of small RNAs termed heterochromatic siRNAs, which are involved in small RNA-directed DNA methylation (RdDM) processes. AGO6 is similar to AGO4 and also interacts with heterochromatic siRNAs. It is involved in RdDM in the growing points of shoots and roots[56]. AGO2 is mainly loaded with *trans*-acting siRNAs (ta-siRNAs) and repeat-associated siRNAs. In addition, AGO7 has been suggested to function in ta-siRNA generation[57,58]. AGO5 contains various siRNAs that originate from intergenic regions[59]. The small RNA content of the remaining *A. thaliana* AGO proteins has not yet been analysed in detail, mainly owing to the highly specific expression patterns in germ cells. Interestingly, AGO9 is expressed in somatic companion cells of female gametes and preferentially interacts with siRNAs derived from transposable elements. It is required for transposable element silencing in the gametes, most probably owing to transport processes between the two cell types[60]. Finally, AGO10 seems to sequester miR-166 and miR-165 specifically and thus regulates shoot apical meristem development[61].

Mammals. In mammals, only one Dicer exists. All four human AGO proteins bind miRNAs and deep-sequencing analysis has shown that distinct miRNA subpopulations might preferentially bind to AGO proteins[62]. However, it has also been reported that miRNAs bind randomly to AGO clade proteins[63,64]. It is possible that the non-catalytic AGO proteins — AGO1, AGO3 and AGO4 — might be at least partially redundant. AGO1 and AGO3 are expressed in many cell lines and tissues, whereas AGO4 expression seems to be less broad[37]. Recent genetic inactivation of mouse AGO4 revealed that it is required for spermatogenesis[65] and that it regulates entry into meiosis. Furthermore, AGO3 has been implicated in *Alu* RNA-guided mRNA decay during stem cell proliferation[66], thus suggesting that non-catalytic mammalian AGO proteins are not redundant. Genetic studies on AGO1 and AGO3 will help to elucidate potential specific functions of these AGO proteins as well. AGO2, which is the only catalytic AGO protein in mammals, is essential for early embryonic development. Its cleavage activity, however, is required only soon after birth, where it processes the highly specialized miRNA miR-451 (REF. 67). In addition to its slicer function, AGO2 binds miRNAs that are partially complementary to target RNAs and engages in gene silencing in a similar manner to AGO1, AGO3 or AGO4.

Many small RNA classes possess specific sequence biases at the 5′ end, and it has become clear that Argonaute proteins are able to sense the 5′-terminal nucleotide of the small RNA. A rigid loop in the MID domain of human AGO2 allows specific contacts to a 5′-terminal uridine or adenine. Small RNAs with Gs or Cs at the 5′ end bind to human AGO2 with low affinities[68]. Similar small-RNA-binding strategies have been found for plant and *D. melanogaster* AGO proteins[54,69].

Endogenous short interfering RNAs
(endo-siRNAs). Short interfering RNAs (siRNAs) that originate from endogenous double-stranded RNA sources, such as long hairpin structures or sense or antisense transcripts from specific genomic loci that form double-stranded RNAs.

Trans-acting siRNAs
(ta-siRNAs). Small RNAs derived from specific genes; the transcripts are cleaved by specialized miRNAs and the cleavage products can be converted into double-stranded RNAs by RNA-dependent RNA polymerases. A Dicer enzyme cleaves the double stranded RNA to ta-siRNAs. ta-siRNAs are involved in DNA methylation.

Repeat-associated siRNAs
Small RNAs that originate from repetitive regions such as transposable elements. These RNAs are involved in silencing transcripts that are produced from repetitive elements.

Shoot apical meristem
A plant tissue that is located at the tip of the shoot axis. It contains undifferentiated cells and gives rise to lateral organs as well as the stem. It can regenerate.

Argonaute-binding partners

Small RNAs guide Argonaute proteins to target RNAs, and Argonautes serve as platforms for interacting proteins that facilitate the major downstream events of gene silencing. Briefly, miRNAs guide AGO clade members to mRNAs, often to binding sites located in the 3′ untranslated region (UTR; for detailed reviews on miRNA function, see REFS 70–72). In cases in which there is perfect complementarity between the miRNA and the target, and if the AGO is catalytic, the mRNA target is cleaved[73]. This process is rare in mammals but is frequently found in plants. In mammals, miRNAs usually bind to partially complementary sites. The so-called 'seed sequence' of the miRNA, comprising nucleotides 2 to 7 or 8, is often fully paired and essential for the interaction. As a consequence of miRNA binding, the mRNA is translationally silenced or degraded[71]. Recently, the dynamics of miRNA-guided gene silencing was investigated in different model systems, and it was found that translational inhibition is the dominant form of repression at early time points, whereas mRNA decay is the major contributor at later time points[74–77].

GW proteins. miRNA-guided gene repression is mediated by and assisted by many additional proteins. For target mRNA repression, AGO proteins directly interact with a member of the GW protein family[19,78–81]. GW proteins are characterized by an N domain containing multiple glycine–tryptophan (GW) repeats. This domain directly interacts with AGO proteins and is therefore referred to as the AGO-binding domain[71]. The recent structure of human AGO2 crystallized in the presence of free tryptophan amino acids, identified two binding pockets for tryptophans on the surface of the PIWI domain, suggesting that GW proteins use only two of their numerous tryptophans for AGO binding[16]. The many GW repeats in the GW proteins suggest that more than one AGO molecule could simultaneously bind to a single GW protein. However, the stoichiometry is unclear, and further biochemical and biophysical studies are required.

The carboxy-terminal half of each GW protein contains several domains and short binding motifs, including an RNA recognition motif (RRM) and a poly(A)-binding protein-interacting motif 2 (PAM2) motif. Both of these motifs interact with poly(A)-binding protein C (PABPC) on the poly(A) tail of the mRNA. It has been proposed that GW binding to PABPC inhibits its interaction with the cap-binding complex and prevents the mRNA from circularization, which is a prerequisite for efficient translational initiation[82,83]. Alternatively, a more direct role of PABPC in miRNA-guided gene silencing has recently been proposed[84]. PABPC and the poly(A) tail stimulate the association of AGO–GW protein complexes to the mRNA, which explains why silencing efficiency correlates with the length of the poly(A) tail and thus with the number of PABPC molecules that are bound to it[84].

Interestingly, the C-terminal silencing domain of GW proteins also contains a short stretch that is enriched in GW repeats. This motif interacts with NOT1, which is a part of the deadenylase CCR4–NOT complex, and recruits it to the mRNA, leading to removal of the poly(A) tail[85,86,87]. A short poly(A) tail leads to decapping of the mRNA and subsequent decay by the abundant 5′ to 3′ exonuclease XRN1 (REF. 71). However, the interaction of miRNA-containing RISC with PABPC is also required for translational repression, supporting a model in which PABPC recruits miRISC to the mRNA without the immediate removal of the poly(A) tail by the deadenylation machinery[88].

Additional AGO interaction partners. Several proteomics studies have sought to identify cellular interaction partners of AGO proteins[19,32,89–92]. In addition to the proteins that are required for RISC loading (for example, HSP90), many proteins with RNA-binding activity are among the binding partners. Many of these proteins are indirectly associated with AGO proteins by binding to the same RNAs. Nevertheless, such proteins can influence the efficiency of miRNA-guided gene silencing. For example, the RNA-binding protein Pumilio (Pum) binds to the 3′ UTR of the *E2F3* mRNA and enhances the activity of several miRNAs that regulate *E2F3* (REF. 93). It is possible that RNA-binding proteins such as Pumilio help to establish a stable interaction of the miRNA with the mRNA[89]. AGO proteins do not directly touch the mRNA, and the base pairing via the short seed region of the miRNA might not be sufficient for stable binding of miRISC to the target mRNA. Other proteins found in biochemical studies, such as importin 8 (IPO8) or UPF1, seem to support AGO complexes in target association[90,94]. In addition, it has been proposed that LIM domain proteins help to stabilize the silenced complex by simultaneously interacting with the 5′ cap-binding complex and AGO proteins [95], and the mammalian hyperplastic disc homologue EDD (also known as UBR5) helps in the recruitment of downstream factors by binding to GW proteins[96]. A protein that is associated with the 40S ribosomal subunit — namely, receptor for activated C kinase 1 (RACK1) — interacts with AGO proteins and recruits miRISC to sites of translation[97].

Proteins that guide RNA-independent recruitment of AGO proteins. It has been found in Dicer knockout mouse embryonic stem cells (ESCs) and in Dicer-deficient mouse embryonic fibroblasts (MEFs), in which mature miRNAs are not produced, that AGO proteins can still associate with mRNA targets[32,98]. These observations suggest that AGO proteins can reach mRNA targets without the guidance of miRNAs. Indeed, it has been observed in *Caenorhabditis elegans* and in human cells that the RNA-binding protein Pumilio/FBF (PUF) helps to recruit AGO proteins to mRNA and a stable complex of PUM, an AGO protein and eukaryotic elongation factor 1α (EF1α1; also known as eEF1A) inhibits translation elongation[99]. In *D. melanogaster*, it was reported that the RNA-binding protein recruits Ago1 to mRNAs independently of miRNAs, leading to silencing of the target mRNA *nanos*[100]. In a speculative model, Ago proteins are recruited to mRNAs by mRNA-binding proteins, and this process is miRNA-independent (FIG. 2). Such a model might also help to explain the many orphan binding sites

Poly(A)-binding protein-interacting motif 2
(PAM2). This motif is present in several proteins and interacts with the poly(A)-binding protein domain, which is found in several proteins, including PABPC1.

LIM domain
Named after the proteins LIN1L, ISL1 and MEC3, LIM domain proteins have been linked to the cytoskeleton, and they have also been implicated in regulating cell growth, migration, cell–cell adhesion and signalling.

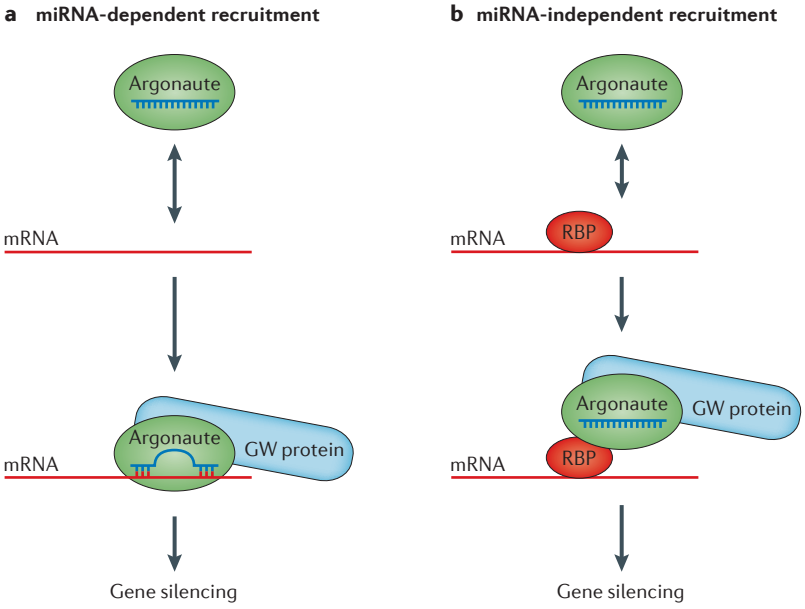


Figure 2 | **A model for the recruitment of AGO proteins to target RNAs.** **a** | MicroRNAs (miRNAs) function as guides to bring AGO proteins to partially complementary sequences located on mRNAs. A member of the GW protein family interacts with the AGO protein and coordinates all of the downstream silencing steps. **b** | AGO proteins can be recruited to target RNAs by protein–protein interaction with RNA-binding proteins (RBPs). In subsequent steps, AGO proteins interact with a member of the GW protein family, which facilitates all downstream silencing processes. Whether binding of small RNAs to AGO proteins is required in such a model is unclear.

found in Ago crosslinking immunoprecipitation experiments (CLIP experiments) that do not match to an miRNA. However, how widespread miRNA-independent Ago targets are has yet to be revealed.

Proteins that associate with PIWI proteins. Owing to their expression mainly in germ cells, few biochemical studies on PIWI clade protein interactors have been carried out. Biochemical purification showed that PIWI proteins interact with the protein arginine *N*-methyltransferase 5 (PRMT5), which symmetrically dimethylates arginine side chains. Indeed, conserved consensus motifs for methylation (the motif GRG) are found in the N domain of PIWI proteins in many organisms, and these are methylated by PRMT5 (REF. 101). It is known from work on splicing that symmetrical methylation of arginines generates a binding platform for Tudor domains[102], and indeed several Tudor domain proteins have been found to associate with dimethylated PIWI proteins[5,103].

Similarly to mouse PIWI proteins, Tudor-domain-containing proteins (TDRDs) exhibit distinct expression patterns during spermatogenesis. The Tudor proteins TDRD1, TDRD2, TDRD4, TDRD6, TDRD7 and TDRD9 are found in mouse germ cells[5]. Many of them might have broader roles in ribonucleoprotein formation or remodelling of such structures in germ cells[104]. Genetic inactivation of TDRD1 and TDRD9 suggests that they have a role during the ping-pong cycle[5], but detailed mechanisms have yet to be unravelled.

In *D. melanogaster*, the Tudor domain proteins Tud, spindle E (SpnE), female sterile (1) Yb (Fs(1)Yb), krimper (Krimp), vreteno (Vret) and tejas (Tej) have also been implicated in Piwi protein binding and function[5,105], but their molecular functions remain elusive. Tud is a large protein containing multiple Tudor domains, and it has been suggested that such proteins might function as platforms that allow for the assembly of higher-order protein–RNA structures. Whether this is true for all Tudor domain proteins, however, is unknown. The main validated direct Argonaute interaction partners are summarized in TABLE 1.

Regulation of Argonaute function

Like most proteins, Argonaute proteins are post-translationally modified, and various types of modification affect Argonaute function (FIG. 3). For example, using mass spectrometry analysis of purified tagged human AGO2, the type I collagen prolyl-4-hydroxylase was found to be an interactor. This enzyme hydroxylates AGO2, and this modification is important for AGO stability[106]. Under cellular stress conditions, human AGO proteins are modified by poly(ADP-ribose), and this modification relieves miRNA-guided repression[107].

Ubiquitylation. AGO proteins are also ubiquitylated. The ubiquitylation identified so far is a degradation signal and is probably more important for AGO turnover than for regulation. TRIM71 (also known as LIN41) has been reported as a potential E3 ubiquitin ligase for AGO2 turnover[108]. Interestingly, a recent report suggests that TRIM71 can function as a direct translational regulator independently of AGO proteins[109]. In addition, in *C. elegans*, a TRIM–NHL family protein called NHL-2 modulates the efficiency of miRISC–mRNA target interaction[110]. In mouse cells, another TRIM–NHL protein, TRIM32, seems to interact with AGO1 and stimulates miRNA activities[111]. Therefore, several TRIM–NHL proteins are linked to the Argonaute machinery, but the mechanisms by which these proteins contribute to Argonaute function are currently unknown.

Polyubiquitylated AGO2 interacts with the autophagy receptor NDP52 (REF. 112). Interestingly, two of the first AGO protein interaction partners to have been identified were GEMIN3 and GEMIN4 (REFS 19,113,114), and these seem to function as adaptor molecules for NDP52 and AGO2 binding[112]. By targeting AGO2, autophagy — which targets cytoplasmic material for destruction — seems to contribute to the homeostasis of miRNA-guided gene silencing[112].

Phosphorylation. Two studies have identified several phosphorylation sites on human Argonaute proteins[115,116]. The phosphorylation events have been implicated in Argonaute protein localization[115] or regulation of small RNA binding[116]. However, clear *in vivo* evidence for the modulation of Argonaute protein function by phosphorylation is still lacking. Signalling cascades that sense intra- or extracellular signals and translate them into gene expression programmes are mainly based on protein phosphorylation. Argonaute proteins are a part of such

Crosslinking immunoprecipitation experiments
(CLIP experiments). CLIP technology facilitates the identification and sequencing of short RNA regions that associate with RNA-binding proteins or with microRNA-induced silencing complexes in intact cells.

Tudor domains
Conserved protein motifs that are able to recognize symmetrically dimethylated arginines.

Table 1 | **Examples of direct Argonaute protein interactors**

Interaction partner	Domains or functions	Function in gene silencing	Refs
AGO clade proteins			
Dicer	RNase III enzyme	Small RNA generation and RISC loading	54
HSP90	Chaperone	RISC loading	54
C3PO (also known as Translin)	Exonuclease complex	RISC activation	41
RHA	Putative helicase	Duplex unwinding	34
MOV10 (mammals)	Putative helicase	Unknown	19
Armitage (*Drosophila melanogaster*)	Putative helicase	RISC formation	36
GW proteins: TNRC6A, TNRC6B and TNRC6C (mammals); Gw182 (*D. melanogaster*); AIN-1 and AIN-2 (*Caenorhabditis elegans*)	Glycine–tryptophan repeats, PAM2 motif, RRM	Coordination of downstream silencing events	71
LIM domain proteins (for example, LIMD1, Ajuba and WTIP)	LIM domain	Stabilization of silencing complexes	95
RACK1	Seven-bladed β-propeller	Ribosome association	97
IPO8	Import receptor	Stabilization of AGO–mRNA interactions	90
UPF1	Helicase, nonsense-mediated decay	Stabilization of AGO–mRNA interactions	94
PUM	RNA-binding protein	AGO recruitment to mRNAs	99
Smg (*D. melanogaster*)	RNA-binding protein	AGO recruitment to mRNAs	100
GEMIN3	Putative helicase	Link AGO proteins to autophagy	112
GEMIN4	GEMIN3 binding partner		
PIWI clade proteins			
Shutdown (*D. melanogaster*)	Co-chaperone	piRNA loading	46
FKBP6 (mouse)	Co-chaperone	piRNA loading	48
PRMT5	Arginine methyltransferase	PIWI protein dimethylation	5
Tudor domain proteins: TDRD1, TDRD 2, TDRD4, TDRD6, TDRD7 and TDRD9 (mouse); Tud, SpnE, Fs(1)Yb, Vret, Krimp and Tej (*D. melanogaster*)	Tudor domain, dimethyl-arginine interaction	Various functions in the germ line	5

Fs(1)Yb, female sterile (1) Yb; HSP90, heat shock protein 90; IPO8, importin 8; Krimp, krimper; PRMT5, protein arginine *N*-methyltransferase 5; PUM, Pumilio; RACK1, receptor of activated C kinase 1; RHA, RNA helicase A; Smg, smaug; SpnE, spindle E; Tej, tejas; TNRC6A, trinucleotide repeat-containing gene 6A protein; Vret, vreteno.

programmes, and it is therefore likely that they are embedded into such signalling pathways. In agreement with such a model, it has been reported that AGO2 expression can be controlled through MAP kinase signalling[117]. Detailed analysis of the interplay between signalling networks and small RNA function will clearly be one of the major future research directions in the gene-silencing field.

Unexpected functions of AGO clade proteins

Using biochemical fractionation and some immunofluorescence staining, AGO clade proteins have been found in the nucleus as well the cytoplasm[90,118–121]. Although increasing amounts of data suggest that AGO clade proteins engage in nuclear processes, nuclear AGO functions in human somatic cells remain unclear. Here, I summarize recent reports on possible nuclear AGO functions (FIG. 4).

AGO clade proteins and transcriptional regulation. In *Schizosaccharomyces pombe*, small RNAs derive from centromeric repeats and associate with the only AGO protein, AGO1, to form the RNA-induced initiation of transcriptional gene silencing complex (RITS)[122]. RITS recruits methyltransferases to specific genomic regions, leading to methylation of histone H3 at lysine 9 (H3K9) and subsequent establishment of silenced heterochromatin (reviewed in REF. 123).

It is also well established that plant AGO proteins, such as AGO4, associate with nascent transcripts and guide small-RNA-directed DNA methylation or chromatin modifications[124–126]. *A. thaliana* AGO4 is found in nuclear foci associated with the nucleolus called Cajal bodies; these bodies are sites of RNP assembly in the nucleus. It has been proposed that a large DNA methylation complex that contains AGO4, siRNAs and possibly many other factors is assembled in Cajal bodies[127,128]. For a detailed review on RNA-mediated chromatin regulation, see REF. 129.

In *C. elegans*, several Argonaute proteins mediate nuclear silencing processes. Early genetic studies revealed that the RNA interference (RNAi) machinery protects the *C. elegans* germ line against transposons[130–132]. More

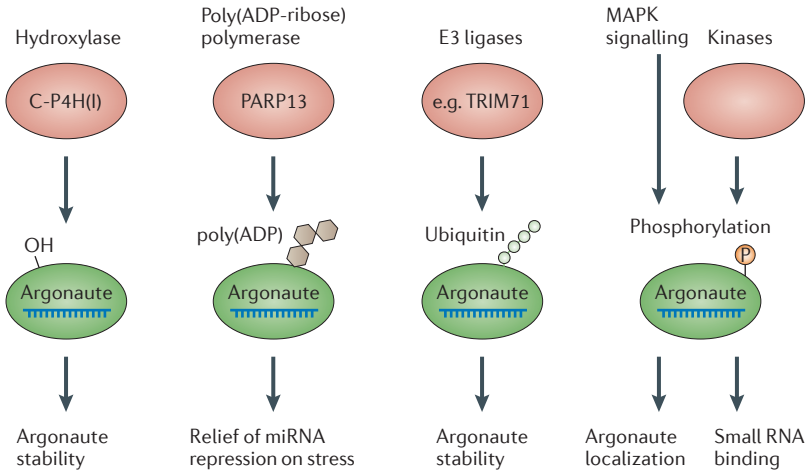


Figure 3 | **Post-translational AGO protein modifications.** AGO proteins can be hydroxylated by the type I collagen prolyl-4-hydroxylase (C-P4H(I)), which affects AGO protein stability. Poly(ADP-ribose) modification on stress leads to the relief of miRNA-guided repression. This modification is added by specific poly(ADP-ribose) polymerases, such as PARP13. Like many other proteins, AGO proteins can be ubiquitylated, resulting in AGO protein degradation. One reported example for a potential AGO-specific E3 ligase is TRIM71. Finally, several phosphorylation sites have been identified on AGO proteins. Phosphorylation at distinct sites seems to be important for correct AGO protein localization or efficient small RNA binding. Mitogen-activated protein kinase (MAPK) signalling has been associated with AGO function. AGO-specific kinases, however, have not yet been reported.

recent work has identified *C. elegans* AGO proteins with distinct nuclear functions. For example, a genetic screen for factors required for nuclear RNAi identified nuclear RNAi-defective 3 (NRDE-3). NRDE-3 is an Argonaute protein that contains a nuclear localization signal (NLS). It is loaded with small RNA in the cytoplasm and transports it into the nucleus, where the complex binds nascent transcripts[133]. Subsequently, NRDE-2, which does not belong to the Argonaute protein family, is recruited and inhibits RNA polymerase II (RNA Pol II) during elongation[134]. Recent work[135] has also implicated an Argonaute protein — heritable RNAi-defective 1 (HRDE-1) — in transmitting the nuclear RNAi signal from parent to progeny in *C. elegans*. HRDE-1 associates with siRNAs in the nucleus of germ cells and directs trimethylation of H3K9. In *hrde-1* mutants, germline silencing is lost over generations. Thus, HRDE-1 and the nuclear RNAi machinery are used to transmit epigenetic information across generations[135].

In *D. melanogaster*, it is also known that heterochromatic silencing and heterochromatin protein 1 (Hp1) localization is dependent on the RNAi machinery, including Ago clade proteins[136,137]. In addition, a recent study found that Ago2 and Dcr2 could associate with transcriptionally active euchromatin and the core transcription machinery. In this study, deep sequencing of Ago2-asociated small RNAs showed that, after heat shock induction, Ago2 is enriched in small RNAs that encompass promoters and other regions of heat shock genes. *Ago2*-null mutants have impaired global RNA Pol II dynamics after heat shock, suggesting a role for Ago2 in controlling RNA Pol II activity[120]. Another study used chromatin immunoprecipitation followed by high-throughput sequencing (ChIP–seq) and confirmed that Ago2 is mainly associated with euchromatin. However, this association is independent of small RNA guides. Ago2 is directly recruited by the chromatin-associated insulator proteins Ctcf and Cf190, and mutations in the genes encoding these proteins lead to a global loss of Ago2 chromatin association[138]. Unlike the situation in *S. pombe*, where RNAi triggers heterochromatin nucleation, *D. melanogaster* nuclear RNAi seems to affect RNA Pol activity at transcriptionally active regions.

RNAi has also been linked to transcriptional silencing in mammals. However, mainly owing to the lack of clear genetic data, AGO-clade-mediated transcriptional gene silencing remains unclear. AGO proteins are found in the nucleus of human cells, and a recent study has suggested that trinucleotide repeat-containing gene 6A protein (TNRC6A), which is a GW protein that is required for miRNA function in the cytoplasm, seems to navigate AGO proteins into the nucleus using its NLS[139]. Early studies showed that AGO proteins interact with the transcription machinery and mediate transcriptional silencing, including chromatin modification[121,140,141]. Subsequently, it was demonstrated that Argonaute-associated small RNAs that are directed against promoter sequences in fact target large sense or antisense transcripts that span the promoter region of distinct genes[142,143]. Such large transcripts are very common and can have activating or inhibiting effects on gene expression. These data suggested a classical RNAi function rather than transcriptional silencing by chromatin modification.

Another interesting twist on nuclear AGO function in human cells came from the analysis of cellular senescence. AGO2 and retinoblastoma 1 (RB1) interact to repress RB1 target genes in senescence; this process has been termed senescence-associated transcriptional gene silencing (SA-TGS). The underlying mechanisms seem to involve silent-state chromatin modifications at the target promoters[144].

AGO clade proteins and alternative splicing. The RNAi machinery has also been implicated in alternative splicing processes in human cells. siRNAs that are directed against exonic or intronic sequences close to an alternative exon regulate alternative splicing[145], and this effect seems to depend on AGO1. Why only this specific AGO protein is required is unknown.

A recent study also found that human AGO1 and AGO2 interact with chromatin modifiers and splicing factors[146]. This work showed that AGO proteins affect RNA polymerase II (RNA Pol II) elongation rates and recruit the spliceosome to splice sites. The mechanism seems to include methylation of H3K9, which slows RNA Pol II, allowing spliceosome assembly[146]. Very recently, *D. melanogaster* Ago2 has also been implicated in alternative splicing events[147].

Although spliceosome composition and dynamics have been characterized in molecular detail[148], Ago proteins or other components of the silencing machinery have not yet been identified in any of the spliceosome preparations. More biochemical work will be required to solidify the model of Ago-mediated alternative splicing.

Spliceosome
A ribonucleoprotein complex that is involved in splicing nuclear precursor mRNA (pre-mRNA). It is composed of five small nuclear ribonucleoproteins (snRNPs) and more than 50 non-snRNPs that recognize and assemble on exon–intron boundaries to catalyse intron processing of the pre-mRNA.

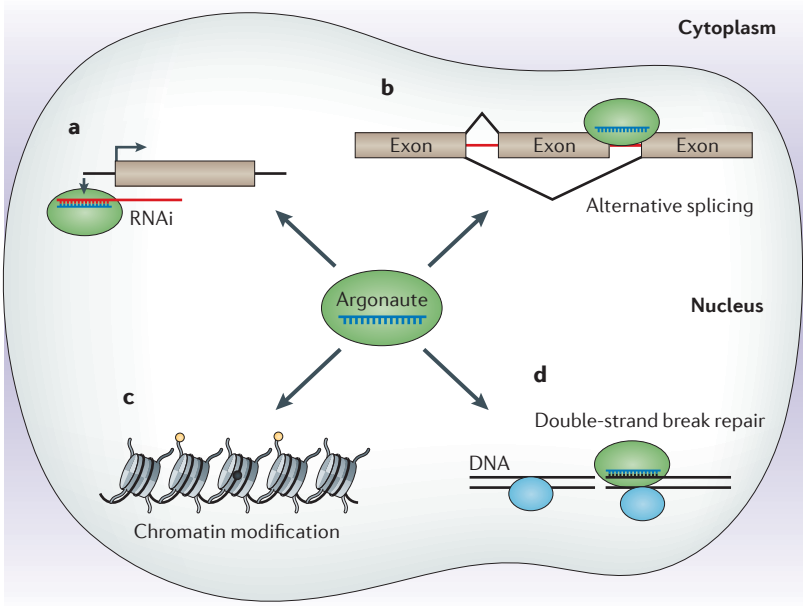


Figure 4 | **Potential nuclear functions of AGO clade proteins. a** | Cleavage-competent AGO proteins can localize to the nucleus, where they can cleave complementary target RNAs in a classical RNA interference (RNAi) mechanisms. In case such RNAs are long non-coding RNAs that regulate gene transcription, cleavage of these RNAs might lead to activation or repression of genes, depending on the function of non-coding RNAs. **b** | One report has recently implicated nuclear AGO clade proteins in alternative splicing processes. **c** | In many organisms, nuclear AGO clade proteins engage in chromatin modification processes, leading to transcriptional silencing. **d** | Small RNAs originating from double-strand break regions have been reported. It has been shown that AGO proteins seem to influence double-strand break repair processes.

AGO clade proteins and double-strand break repair mechanisms. DNA double-strand breaks frequently occur in eukaryotic cells, and various mechanisms have evolved to repair the DNA breaks. In *A. thaliana*, small RNAs are produced from the vicinity of the double-strand breaks and are referred to as double-strand-break-induced small RNAs (diRNAs). Interestingly, such small RNAs are also found in human HeLa cells, and in both plants and humans, they are associated with AGO2. Both Dicer and AGO2 affect double-strand break repair efficiency, suggesting an active role of these factors in this process[149]. Supporting this idea, deep sequencing from *D. melanogaster* cells in which a double-strand break has been induced in a reporter construct identified small RNAs originating from regions flanking the break[150]. Another study found that double-strand break repair requires Dicer and Drosha in human cells but does not require GW proteins. The requirement of AGO proteins was not tested in this study[151]. Many questions remain about this intriguing link between AGO proteins and double-strand break repair. Not least, it is not known how small RNAs are generated from the break locus.

Conclusions

Intensive research over the past 10 years has elucidated the main principles of Argonaute protein function. However, there are still major gaps in our understanding of Argonaute function on the molecular level. For example, more structural work will be required to understand how loading of Argonaute proteins is facilitated. How do Argonaute proteins find their target RNAs, and how are Argonaute proteins removed from their targets when repression is relieved? Furthermore, are there specific Argonaute-unloading pathways? Experimental work into this direction has just been started[152].

Another emerging area that has not yet been studied in depth is the crosstalk between signalling pathways and gene-silencing processes. Argonaute proteins and other factors that are required for small RNA function might be a part of phosphorylation cascades leading to increased or reduced activities. It is becoming increasingly apparent that Argonaute proteins are parts of large regulatory networks. The networks themselves and the dynamics of such networks during cell differentiation or signal transduction are poorly understood. Subtle changes in these networks might lead to mild but chronic effects on gene expression that might ultimately lead to diseases such as cancer. Furthermore, long non-coding RNAs can be a part of these networks as well. Competition of coding and non-coding RNAs for Argonaute binding can influence gene silencing[153]. Therefore, a detailed understanding of Argonaute proteins on both the molecular and cellular levels will help to unravel the principles and causes of diseases.

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