Argonaute proteins at a glance

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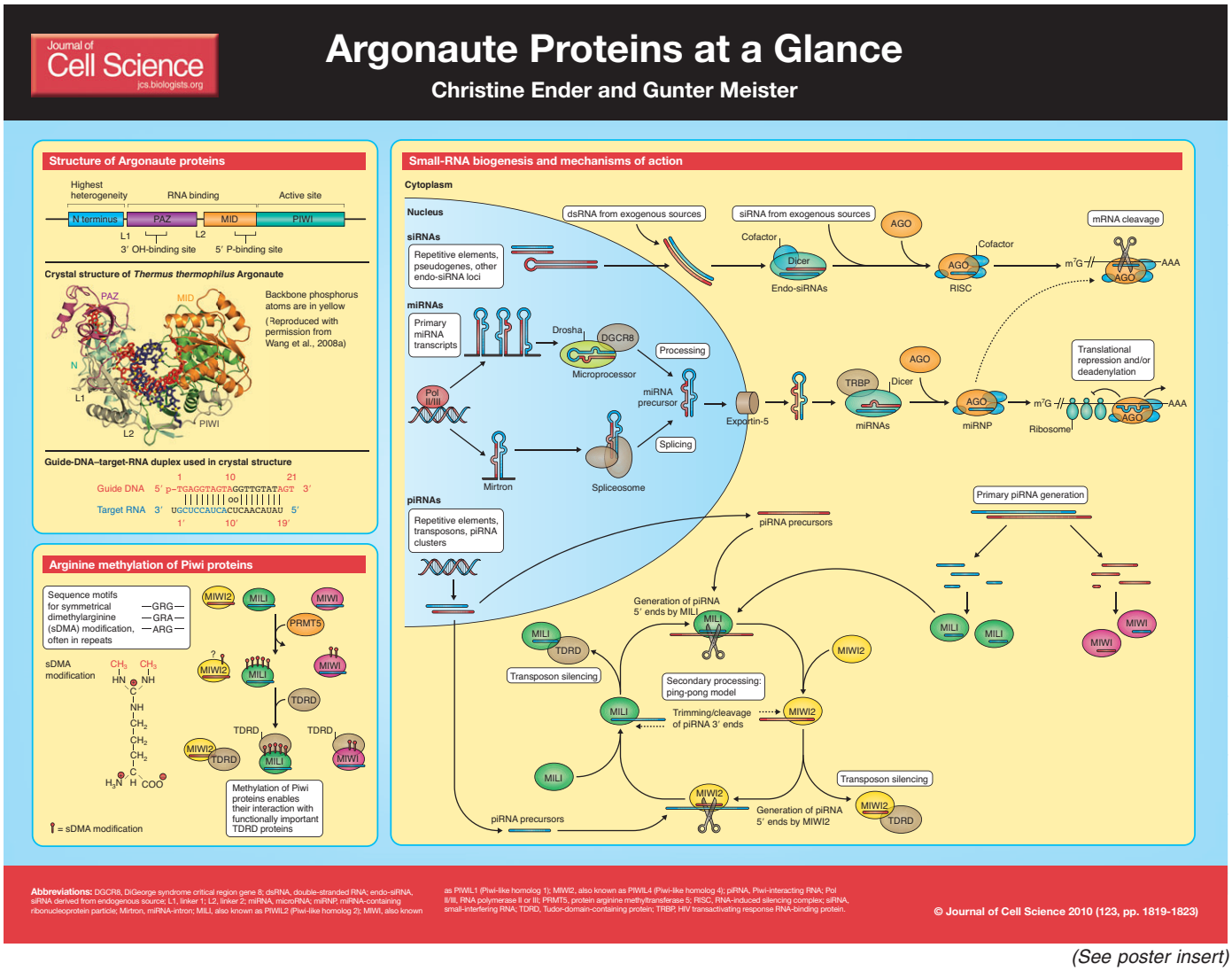
Although a large portion of the human genome is actively transcribed into RNA, less than 2% encodes proteins. Most transcripts are non-coding RNAs (ncRNAs), which have various cellular functions. Small ncRNAs (or small RNAs) – ncRNAs that are characteristically ~20-35 nucleotides long – are required for the regulation of gene expression in many different organisms. Most small RNA species fall into one of the following three classes: microRNAs (miRNAs), short-interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) (Carthew and Sontheimer, 2009). Although different small RNA classes have different biogenesis pathways and exert different functions, all of them must associate with a member of the Argonaute protein family for activity. This article and its accompanying poster provide an overview of the different classes of small RNAs and the manner in which they interact with Argonaute protein family members during small-RNA-guided gene silencing. In addition, we highlight recently uncovered structural features of Argonaute proteins that shed light on their mechanism of action.

Biogenesis of miRNAs and siRNAs

miRNAs

Generally, miRNAs are transcribed by RNA polymerase II or III to form stem-loop-structured primary miRNA transcripts (pri-miRNAs). pri-miRNAs are processed in the nucleus by the microprocessor complex, which contains the RNAse III enzyme Drosha and its DiGeorge syndrome critical region gene 8 (DGCR8) cofactor. The transcripts are cleaved at the stem of the hairpin to produce a stem-loop-structured miRNA precursor (pre-miRNA) of ~70 nucleotides. After this first processing step, pre-miRNAs are exported into the cytoplasm by exportin-5, where they are further processed by the RNAse III enzyme Dicer and its TRBP (HIV transactivating response RNA-binding protein) partner. Dicer produces a small double-stranded RNA (dsRNA) intermediate of ~22 nucleotides with 5′ phosphates and 2-nucleotide 3′ overhangs. In subsequent



(See poster insert)

processing and unwinding steps, one strand of the dsRNA intermediate is incorporated into an Argonaute-protein-containing complex that is referred to as an miRNA-containing ribonucleoprotein particle (miRNP). The opposite strand, the so-called miRNA* (miRNA 'star') sequence, is thought to be degraded. In some cases, however, miRNA* sequences can also be functional (Packer et al., 2008). Mature miRNAs guide Argonaute-containing complexes to target sites in mRNAs that are partially complementary to the miRNA sequence, and induce repression of gene expression at the level of mRNA stability or translation (see poster).

In addition to this canonical miRNA biogenesis pathway, some alternative miRNA biogenesis pathways have recently been discovered. So-called mirtrons (miRNA-introns) are miRNAs found in introns. They are identical to pre-miRNAs with respect to their size and features, but are generated independently of Drosha (Kim et al., 2009; Siomi and Siomi, 2009). Moreover, some classes of small nucleolar RNAs (snoRNAs) can be processed into small RNAs that act like miRNAs (Ender et al., 2008; Scott et al., 2009; Taft et al., 2009).

siRNAs

There are many similarities between the pathways by which miRNAs and siRNAs are processed. In contrast to miRNAs, however, siRNAs are processed independently of Drosha from long dsRNAs that are derived from either exogenous sources (to form exo-siRNAs) or endogenous sources (to form endo-siRNAs). Depending on the organism, exo-siRNAs can be produced through the cleavage of viral RNAs or by introducing long, perfectly base-paired dsRNA into the cytoplasm. Both Dicer and TRBP are required for the processing of siRNAs from long dsRNAs that are derived from exogenous sources. Endo-siRNAs have been reportedly expressed in plants and in animals such as *Caenorhabditis elegans*, flies and mice. Endo-siRNAs are derived from transposable elements, natural antisense transcripts, long intermolecularly paired hairpins and pseudogenes (Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008).

After Dicer processing, siRNA duplexes are separated and one strand is incorporated into the RNA-induced silencing complex (RISC). The single-stranded RNA, often referred to as the guide strand (the other strand is known as the passenger strand), directs RISC to perfectly complementary sites in target mRNA molecules; RISC then cleaves the target mRNA (Kim et al., 2009).

The Argonaute protein family

To perform their effector functions, small RNAs must be incorporated into Argonaute-protein-containing complexes. Argonaute proteins are highly specialized small-RNA-binding modules and are considered to be the key components of RNA-silencing pathways. Argonaute proteins were named after an AGO-knockout phenotype in *Arabidopsis thaliana* that resembles the tentacles of the octopus *Argonauta argo* (Bohmert et al., 1998). On the basis of sequence homology, Argonaute proteins can be divided into two subclasses. One resembles *Arabidopsis* AGO1 and is referred to as the Ago subfamily; the other is related to the *Drosophila* PIWI protein and is referred to as the Piwi subfamily.

Members of the human Ago subfamily, which consists of AGO1, AGO2, AGO3 and AGO4, are ubiquitously expressed and associate with miRNAs and siRNAs. Ago proteins are conserved throughout species and many organisms express multiple family members, ranging from one in *Schizosaccharomyces pombe*, five in *Drosophila*, eight in humans, ten in *Arabidopsis* to twenty-seven in *C. elegans* (Tolia and Joshua-Tor, 2007). Argonaute proteins are also present in some species of budding yeast, including *Saccharomyces castellii*. It was recently found that *S. castellii* expresses siRNAs that are produced by a Dicer protein that differs from the canonical Dicer proteins found in animals, plants and other fungi (Drinnenberg et al., 2009). However, the model organism *Saccharomyces cerevisiae* lacks Argonaute proteins and none of the known small RNA pathways are conserved in *S. cerevisiae*. Argonaute proteins are also found in some prokaryotes (Jinek and Doudna, 2009), but their function in these organisms remains unclear.

The expression of Piwi subfamily members is mainly restricted to the germline, in which they associate with piRNAs. The human genome encodes four Piwi proteins, named HIWI (also known as PIWIL1), HILI (also known as PIWIL2), HIWI3 (also known as PIWIL3) and HIWI2 (also known as PIWIL4) (Peters and Meister, 2007). There are three Piwi proteins in mice, known as MIWI, MILI and MIWI2.

Structure and function of Argonaute proteins

Small RNAs regulate gene expression by guiding Argonaute proteins to complementary sites on target RNA molecules. Recent structural studies of bacterial and archaeal Argonaute proteins have shed light on the mechanism of silencing mediated by both Ago and Piwi subfamily proteins.

Argonaute proteins typically have a molecular weight of ~100 kDa and are characterized by a Piwi-Argonaute-Zwille (PAZ) domain and a PIWI domain. Crystallographic studies of archaeal and bacterial Argonaute proteins revealed that the PAZ domain, which is also common to Dicer enzymes, forms a specific binding pocket for the 3′-protruding end of the small RNA with which it associates (Jinek and Doudna, 2009). The structure of the PIWI domain resembles that of bacterial RNAse H, which has been shown to cleave the RNA strand of an RNA-DNA hybrid (Jinek and Doudna, 2009). More recently, it was discovered that the catalytic activity of miRNA effector complexes, also referred to as Slicer activity, resides in the Argonaute protein itself. Interestingly, not all Argonaute proteins show endonucleolytic activity. In humans, only AGO2 has been shown to cleave the phosphodiester bond of a target RNA, at a site opposite nucleotides 10 and 11 of the siRNA, although the conserved aspartic acid-aspartic acid-histidine (DDH) motif (which is important for divalent metal ion binding and catalytic activity) is also present in human AGO3 (Liu et al., 2004; Meister et al., 2004).

More recently, structural studies have been extended to *Thermus thermophilus* Argonaute in complex with a guide strand only or a guide DNA strand and a target RNA duplex. This analysis revealed that the structure of the complex is divided into two lobes. One lobe contains the PAZ domain connected to the N-terminal domain through a linker region, L1. The second lobe consists of the middle (MID) domain (located between the PAZ and the PIWI domains) and the PIWI domain. The 5′ phosphate of the small RNA to which Argonaute binds is positioned in a specific binding pocket in the MID domain (Jinek and Doudna, 2009). The contacts between the Argonaute protein and the guide DNA or RNA molecule are dominated by interactions with the sugar-phosphate backbone of the small RNA or DNA; thus, the bases of the RNA or DNA guide strand are free for base pairing with the complementary target RNA. The structure indicates that the target mRNA base pairs with the guide DNA strand, at least in the region of the seed sequence (which is especially important for target recognition), but does not touch the protein (Wang et al., 2008a; Wang, Y. et al., 2009; Wang et al., 2008b).

Although significant progress has been made regarding the structure of bacterial and archaeal Argonaute proteins, no structures of mammalian Argonaute proteins are available. As bacterial Argonaute proteins have a high affinity for short DNA guide strands, it will be interesting to see how mammalian

Argonaute proteins interact with short RNA guide strands.

miRNAs and siRNAs guide Ago proteins to their target mRNA. A key determinant of the regulatory mechanism of RNA silencing is the degree of complementarity between the small RNA and the target mRNA. Perfect complementarity promotes AGO2-mediated endonucleolytic cleavage, whereas mismatches in the central region of the small RNA lead to repression of gene expression at the level of translation or mRNA stability. However, the mechanisms underlying translational repression are not yet clear. Studies from several laboratories have provided support for the suggestion that miRNA-mediated repression occurs at early steps of translation. Using density-gradient fractions, it was shown that mRNAs repressed by miRNAs do not sediment in polysome fractions, but shift to the free messenger ribonucleoprotein pool. Furthermore, it was shown that miRNA-mediated translational repression could only target mRNAs containing a functional m^7G cap (Filipowicz et al., 2008; Pillai et al., 2007). It has therefore been suggested that miRNA-guided translational repression is based on preventing the circularization of the mRNA that is needed for stimulation of translation. Contradicting this model are studies in which mRNA transcripts lacking a poly(A) tail can still be targeted by miRNA-mediated repression (Carthew and Sontheimer, 2009; Filipowicz et al., 2008; Pillai et al., 2007). In contrast to the translation initiation model of miRNA function, evidence of miRNA-mediated effects during later stages of translation has been reported. It has been suggested that miRNA causes mRNAs to prematurely dissociate from or 'drop off' ribosomes. Furthermore, an independent model proposes that the nascent polypeptide chain translated from the mRNA target is degraded co-translationally (Carthew and Sontheimer, 2009; Filipowicz et al., 2008).

In addition to their capacity to mediate translational repression, it has been demonstrated that miRNAs can guide the destabilization of target mRNAs that contain imperfectly complementary target sites (Bagga et al., 2005; Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wu and Belasco, 2005). The mechanism of miRNA-mediated mRNA decay requires proteins of the mRNA-degradation machinery and depends on a member of the Ago protein family and a member of the GW182 protein family (Behm-Ansmant et al., 2006; Eulalio et al., 2008b; Jakymiw et al., 2005; Liu et al., 2005; Meister et al., 2005). GW182 is characterized by the presence of glycine and tryptophan repeats (GW repeats) and localizes to processing bodies (P-bodies), cytoplasmic regions enriched for proteins involved in mRNA turnover (Eulalio et al., 2007). Insects have one GW182 protein, *C. elegans* has two (AIN1 and AIN2) and humans have three paralogs (TNRC6A/GW182, TNRC6B and TNRC6C) (Eulalio et al., 2007). GW182 proteins associate with Ago proteins through direct protein-protein interactions. It has been demonstrated that some GW motifs form Ago-interaction platforms that interact with the MID domain of Ago proteins. Such GW repeats are referred to as Ago 'hooks' (Till et al., 2007). In *Drosophila*, depletion of GW182 leads to increased levels of mRNAs targeted by miRNAs (Behm-Ansmant et al., 2006; Eulalio et al., 2008a). Moreover, tethering of GW182 to a target mRNA represses translation of the mRNA independently of *Drosophila* AGO1, demonstrating that GW182 is an important effector protein functioning downstream of Ago proteins (Behm-Ansmant et al., 2006). A recent study in Krebs-2 mouse ascite cell extract (Fabian et al., 2009) and an earlier study in *Drosophila* (Behm-Ansmant et al., 2006) found that CAF1, a component of the CCR-NOT deadenylase complex that is required for the removal of the poly(A) tail of mRNAs, is at least partially responsible for miRNA-mediated deadenylation of target mRNAs. Furthermore, poly(A)-binding protein (PABP), which interacts with the poly(A) tail of mRNAs and the eukaryotic translation initiation factor 4G (eIF4G) subunit of the cap-binding complex that circularizes the mRNA during translational initiation, also interacts with GW182 proteins (Beilharz et al., 2009; Fabian et al., 2009). Consistently, it has been proposed that GW182 proteins might compete with eIF4G for PABP binding and therefore prevent mRNA circularization. Uncircularized mRNA might not be able to initiate translation efficiently and protein expression is reduced (Beilharz et al., 2009; Fabian et al., 2009; Zekri et al., 2009).

In summary, Ago proteins are highly specialized small-RNA-binding proteins. Small RNAs guide Ago proteins to complementary target mRNAs, where Ago proteins act together with protein binding partners to interfere with translation or induce deadenylation of target mRNAs.

Piwi proteins, piRNA function and transposon silencing

In *Drosophila*, PIWI, Aubergine (AUB) and AGO3 constitute the Piwi subfamily. Mutations in the PIWI protein lead to defects in oogenesis and depletion of germline cells, whereas AUB mutations disrupt gametogenesis. In mice, all Piwi proteins are important for spermatogenesis. The discovery of piRNAs, the small RNA partners to which Piwi proteins bind, helped to further elucidate the function of these proteins (Aravin and Hannon, 2008). In mammals, piRNA expression changes during stages of sperm development. During the pre-pachytene stage of meiosis, piRNAs predominantly correspond to repetitive and transposon-rich sequences, and interact with mouse Piwi subfamily members MILI and MIWI2. piRNAs found during the pachytene stage of meiosis associate with Piwi subfamily members MILI and MIWI (Aravin and Hannon, 2008). In mice, it has been demonstrated that piRNAs act together with their respective Piwi protein partners to repress the expression of mobile genetic elements in the germline. Such mobile elements can randomly integrate into the genome and it is important that such events are repressed in the germline. Recently, it has been found that Piwi proteins are methylated by the arginine methyltransferase PRMT5 and possibly by other methyltransferases. These post-translational modifications enable Piwi proteins to interact with Tudor-domain-containing proteins (TDRDs) (Kirino et al., 2009; Reuter et al., 2009; Vagin et al., 2009). Not much is known about the function of TDRD proteins in gene silencing. It has been suggested that TDRD1 contributes to pre-pachytene piRNA biogenesis and is important for silencing repetitive elements.

In addition to piRNAs, the endo-siRNA pathway also contributes to transposon repression (Okamura and Lai, 2008). In the male germline, mutations in components of the piRNA pathway have a severe effect on fertility, whereas this pathway appears to be dispensable in the female germline of mammals, which is enriched in endo-siRNAs (Tam et al., 2008; Watanabe et al., 2008). It might therefore be possible that, in the germline, the piRNA pathway and the endo-siRNA pathway cooperate with each other in transposon repression.

Although thousands of individual piRNA sequences have been identified, they derive from discrete genomic clusters (Aravin and Hannon, 2008). *Drosophila* Piwi subfamily members PIWI and AUB bind to piRNAs that are antisense to transposon RNAs, whereas the third Piwi protein in flies, AGO3, typically binds the sense strands. AUB- and PIWI-associated piRNAs preferentially carry a uracil at their 5′ end, whereas most AGO3-associated piRNAs have an adenine at nucleotide 10. With the discovery that the first ten nucleotides of antisense piRNAs are often complementary to the sense piRNAs, the 'ping-pong' model has been proposed (Kim et al., 2009). In this model, AUB or PIWI proteins that are associated with antisense piRNAs cleave sense retrotransposon transcripts, thereby creating the 5′ end of sense piRNAs that then associate with AGO3. AGO3

subsequently cleaves antisense retrotransposon transcripts, generating the 5′ end of antisense piRNAs that subsequently bind to PIWI. It is not known how the 3′ end of the piRNA is generated. However, it is tempting to speculate that exonucleases or endonucleases interact with Piwi proteins, and either trim or cleave the RNA to generate the correct piRNA 3′ end. Through this continuous cycle, piRNAs are amplified and retrotransposon silencing can be maintained. However, the mechanism by which piRNA biogenesis is initiated is not understood. It is known that AUB and PIWI are maternally inherited (Brennecke et al., 2008). It is possible that the maternally contributed piRNAs that are associated with AUB and PIWI act as primary piRNAs and initiate the ping-pong cycle in the embryo. The ping-pong model found in *Drosophila* might also be applicable to mouse piRNAs and the mouse Piwi proteins MILI and MIWI2.

Argonaute proteins in the nucleus

In addition to their role in small-RNA-mediated silencing at the mRNA level in the cytoplasm, Argonaute proteins are also thought to function in the nucleus at the transcriptional level. Transcriptional gene silencing was first discovered in plants, in which small RNAs derived from transgenes and viral RNAs were found to guide methylation of homologous DNA sequences. Further studies showed that DNA methylation of a specific transgene was Dicer and Argonaute dependent, and linked to histone H3 lysine 9 (H3K9) methylation. In contrast to plants, small RNAs in *S. pombe* can only induce histone methylation and are not capable of guiding DNA-methylation events. The AGO1-containing effector complex in *S. pombe* is termed the RNA-induced transcriptional silencing (RITS) complex, which associates with nascent transcripts and the DNA-dependent RNA polymerase. A histone methyltransferase leads to H3K9 methylation and subsequent heterochromatin formation (Moazed, 2009).

In mammals, siRNAs that are directed to gene promoters can induce histone methylation dependent on AGO1 (and AGO2) (Janowski et al., 2006; Kim et al., 2006). Furthermore, a role for promoter-directed human miRNAs in facilitating transcriptional gene silencing has been described (Kim et al., 2008). Promoter-targeting RNAs have also been found to be involved in activating transcription (Janowski et al., 2007; Schwartz et al., 2008). These results might suggest that small-RNA-mediated DNA and histone modifications also occur in mammals. However, for a conclusive model of how Argonaute proteins function on mammalian chromatin, further experimental work is needed. Further supporting the idea that small-RNA pathways are functional in the nucleus, it has also recently been reported that Ago proteins localize to the nucleus (Weinmann et al., 2009). However, the mechanistic details of small-RNA function in the nucleus of mammalian cells remain unclear.

Perspectives

Although many aspects of Argonaute function have been characterized, there are still many open questions. Why do only some Argonaute proteins possess endonuclease activity, despite containing crucial conserved amino acids? Two recent publications have reported that Argonaute proteins might have different cleavage preferences: whereas only human AGO2 cleaves complementary target mRNAs through an RNAi-like mechanism, both AGO1 and AGO2 can cleave the passenger strand of an siRNA duplex (Steiner et al., 2009; Wang, B. et al., 2009). However, further work will be necessary to confirm these initial observations. It is also becoming increasingly apparent that Argonaute proteins can be post-translationally modified: modifications such as hydroxylation, phosphorylation and ubiquitylation influence Argonaute stability and function in mammals (Qi et al., 2008; Rybak et al., 2009; Zeng et al., 2008). However, Argonaute modifications have not yet been analyzed systematically. Future research will not only unravel novel Argonaute modifications, but will also help to place Argonaute proteins within complex cellular signaling pathways. Such findings might also help to understand the molecular basis of various diseases in which gene expression is disrupted, including cancer.

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