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The RNA-induced Silencing Complex: A Versatile Gene-silencing Machine*

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Ashley J. Pratt and Ian J. MacRae[1]
From the Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

RNA interference is a powerful mechanism of gene silencing that underlies many aspects of eukaryotic biology. On the molecular level, RNA interference is mediated by a family of ribonucleoprotein complexes called RNA-induced silencing complexes (RISCs), which can be programmed to target virtually any nucleic acid sequence for silencing. The ability of RISC to locate target RNAs has been co-opted by evolution many times to generate a broad spectrum of gene-silencing pathways. Here ,we review the fundamental biochemical and biophysical properties of RISC that facilitate gene targeting and describe the various mechanisms of gene silencing known to exploit RISC activity.

RISC[2] is a generic term for a family of heterogeneous molecular complexes that can be programmed to target almost any gene for silencing. In general, RISC programming is triggered by the appearance of dsRNA in the cytoplasm of a eukaryotic cell (Fig. 1). The dsRNA is processed into small regulatory RNAs (20–30 nucleotides in length) that assemble into RISC and guide the complex to complementary RNA targets through base-pairing interactions. Once programmed with a small RNA, RISC can silence targeted genes by one of several distinct mechanisms, working at (*a*) the level of protein synthesis through repression of translation, (*b*) the transcript level through mRNA degradation, or (*c*) the level of the genome itself through the formation of heterochromatin or by DNA elimination.

Although the mechanisms used to control gene expression by RISC are quite diverse, two central themes are common to all. First, at its core, every RISC contains a member of the Argonaute protein family that binds to the small regulatory RNA. Second, in every RISC, the small regulatory RNA functions as a guide that leads RISC to its target through Watson-Crick base pairing with cognate RNA transcripts. The role of the Argonaute protein is to bind the small RNA and position it in a conformation that facilitates target recognition. Argonaute proteins can either cleave target RNAs directly or recruit other gene-silencing proteins to identified targets. Here, we review how Argonaute proteins use small RNAs to recognize target transcripts. We also examine how recruitment of different types of Argonaute and Argonaute-associated proteins produce distinct RISCs, which then dictate the mechanism of gene regulation.

RISC and Small RNA Nomenclature

As in many fields of biology, the nomenclature commonly used to describe RNAi and RISC is not completely rational or intuitive. The small regulatory RNAs that guide RISC have been given a variety of similar sounding names. These include siRNA, miRNA, piRNA, rasiRNA, tasiRNA, tncRNA, hcRNA, and scnRNA. These classifications are generally based on either the biosynthetic pathway of the small RNA or the type of RISC in which the RNA is found (for a detailed review, see Ref. 1). However, once bound to an Argonaute protein, all small regulatory RNAs function in the same way: as guides for gene silencing through base-pairing interactions with target RNA transcripts.

Another point of potential confusion is that an exact molecular composition for RISC has never been defined, and furthermore, the term RISC has been used to describe several biochemically distinct gene-silencing complexes. The minimal RISC, sufficient for target RNA recognition and cleavage, was demonstrated to be simply an Argonaute protein bound to a small RNA (2). However, Argonaute proteins can have dozens of associated binding partners, which do not assemble as one distinct complex *in vivo*. Biochemical isolations of RISC have uncovered a variety of different RNPs, ranging from modest size (~150 kDa) (3) up to an 80 S (~3 MDa) particle termed "holo-RISC" (4) and many other intermediate sizes (5–8). Additional names given to these complexes include siRISC and miRISC, RISCs in *Drosophila* that contain either an siRNA or miRNA, respectively (9); miRNP, an miRNA-containing RNP in HeLa cells likely similar to miRISC (10); and the RITS (RNA-induced transcriptional gene silencing) complex, isolated from *Schizosaccharomyces pombe* nuclei and shown to silence targeted genes through heterochromatin formation (11). The underlying feature common to all of these silencing complexes is that the core of each contains a small regulatory RNA guiding an Argonaute protein. Therefore, insight into the mechanism of RISC and the details underlying gene silencing by RNAi depends on an understanding of the Argonaute proteins.

Argonaute Proteins Are Abundant in Nature

Argonaute proteins are ubiquitous in plants and animals, common in many fungi and protists, and also present in some archaea. The number of Argonaute genes found in these different species varies from one (as in the fission yeast *S. pombe*) to over two dozen (27 in *Caenorhabditis elegans*). In some cases, multiple copies of an Argonaute gene are functionally redundant. For example, in *C. elegans*, Argonaute proteins ALG-1 and ALG-2 are sufficient to recompense for one another (12). However, it is common for the Argonaute genes in an organism to be specialized and have non-overlapping functions.

The eukaryotic Argonaute family can be classified into three major phylogenetic clades based on amino acid sequence similarities (1). The largest clade is named Argonaute (for clarity, we refer

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[1] Pew Scholar in the Biomedical Sciences. Supported by Grant 1RO1GM086701 from the National Institutes of Health. To whom correspondence should be addressed. E-mail: macrae@scripps.edu.

[2] The abbreviations used are: RISC, RNA-induced silencing complex; dsRNA, double-stranded RNA; RNAi, RNA interference; siRNA, small interfering RNA; miRNA, microRNA; piRNA, PIWI-interacting RNA; scnRNA, small scan RNA; RNP, ribonucleoprotein; P-bodies, processing bodies.

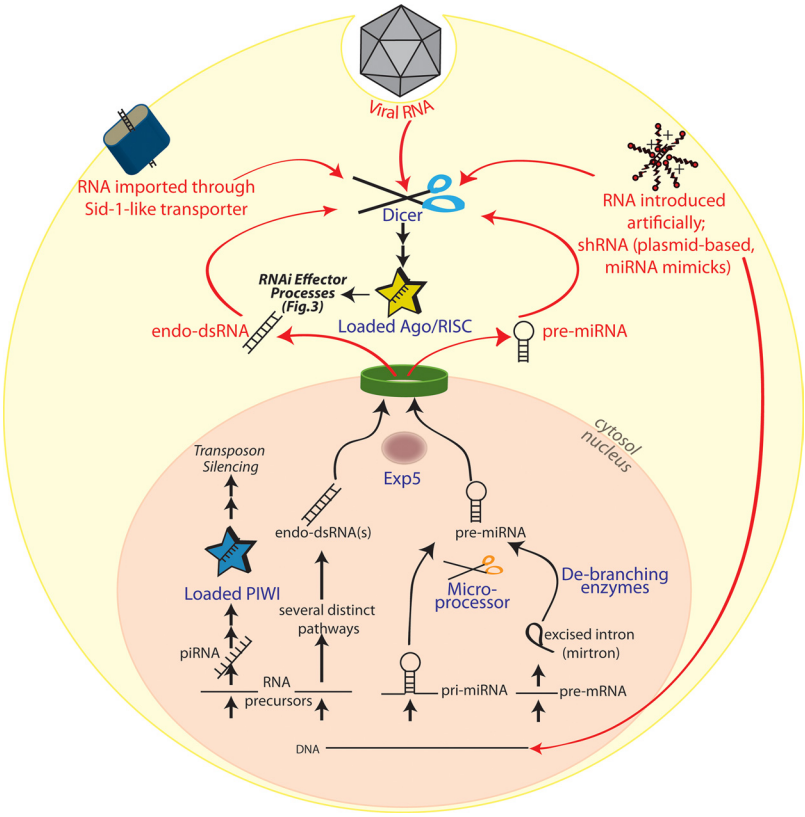

FIGURE 1. **Formation of RISCs and other silencing complexes.** Silencing RNA can be derived from exogenous or intracellular origins, depending on the organism and cell type. RNA can also be introduced artificially using siRNA or plasmid-based short hairpin RNA (*shRNA*) systems. RNAs transcribed from the genome may be retained in the nucleus (as with piRNAs) to carry out silencing or may be exported (as with miRNAs). In the cytoplasm, dsRNA is processed by the endonuclease Dicer and loaded onto an Argonaute protein, and after the strand selection process, the newly formed RISC is equipped to silence target genes by one of several mechanisms.

to class this class as Ago) after its founding member AGO1 in *Arabidopsis* (13). At the cellular level, Ago proteins localize diffusely in the cytoplasm and nucleus and, in some cases, also at distinct foci, which include P-bodies and stress granules (14, 15). The second clade, Piwi (named after the *Drosophila* protein PIWI, for P-element-induced wimpy testis), is most abundantly expressed in germ line cells and functions in the silencing of germ line transposons (16). The human genome contains four copies of both *AGO* and *PIWI* genes. A diverse assortment of nematode-specific Argonaute genes has been grouped together into a third clade called class 3 or worm Argonautes (WAGOs) (17).

A major biochemical difference between Argonaute clades is the means by which members acquire guide RNAs. Ago guide RNAs are generated from dsRNA in the cytoplasm by a specialized nuclease named Dicer (Fig. 1). Members of the Piwi clade are thought to form guide RNAs in a "ping-pong" mechanism in which the target RNA of one Piwi protein is cleaved and becomes the guide RNA of another Piwi protein (18). Maternally inherited guide piRNAs are believed to initiate this gene-silencing cascade (19). Class 3 Argonautes obtain guide RNAs by Dicer-mediated cleavage of exogenous and endogenous long dsRNAs (17).

Structure of Argonaute: The Heart of RISC

At present, structural information describing eukaryotic Argonautes is sparse. However, crystal structures of several prokaryotic Argonautes have been described (Ref. 20 and references therein). Prokaryotic Argonautes bind small guide DNAs and can use these guides to locate and cleave target RNAs *in vitro*, in a manner analogous to the small RNA-guided function of their eukaryotic cousins (21). The biological functions of Argonaute proteins and small DNAs in the prokaryotic kingdom have yet to be discovered. However, structures of the prokaryotic Argonautes have provided a wealth of information about the overall Argonaute architecture and mechanism.

Prokaryotic Argonaute proteins adopt a bilobed structure, with each lobe responsible for binding opposite ends of the guide DNA (Fig. 2). The N-terminal lobe contains a PAZ domain, which binds the 3′-end of the small guide DNA. The C-terminal lobe contains a middle domain, which binds to the 5′-phosphate of the guide DNA, and the PIWI domain, whose terminal carboxyl group interacts with the 5′-phosphate via coordination of a divalent cation. The PIWI domain adopts a RNase H-like fold and can hydrolyze target RNAs using an RNase H-like mechanism (22) This activity has been dubbed "slicing" of target RNAs. A flexible hinge composed of a two-stranded β-sheet connects the two lobes. Flexibility in the hinge allows the lobes to pivot relative to each other, opening a cleft to accommodate the guide DNA and RNA target (20, 23).

Guide Binding Interactions

The 5′-phosphate of the guide DNA is held in a pocket between the middle and PIWI domains, which acts as an anchoring point for the DNA in the protein. In eukaryotes, the 5′-phosphate licenses small RNAs for entry into the RNAi pathway (7). The first nucleotide base on the 5′-end also tucks into a small defined binding pocket. This allows the protein to make base-specific contacts with the first nucleotide in the guide, which may explain why some eukaryotic Argonautes have preferences for particular bases on the 5′-end (24). It also explains why the first base in the guide RNA does not contribute significantly to target recognition (25). The rest of the DNA contacts the protein almost exclusively through its phosphodiester backbone, explaining how Argonaute can bind to any guide strand, regardless of nucleotide sequence. The two terminal bases on the 3′-end are clamped into a hydrophobic cleft in the PAZ domain. Interestingly, whereas the ends of the guide are tightly bound to Argonaute, bases 11–18 are disordered in the crystal structure, suggesting a high degree of mobility for this part of the guide (23).

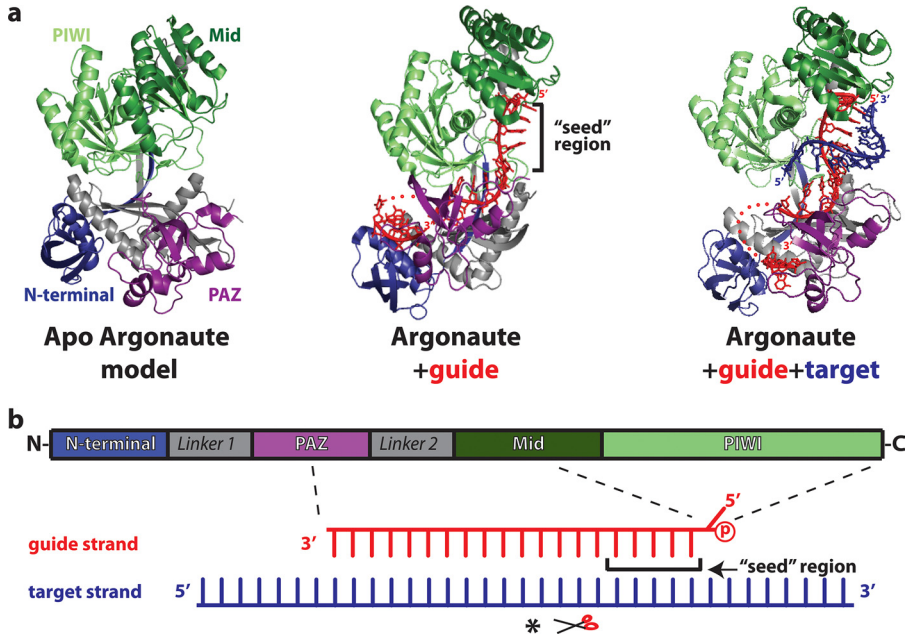

FIGURE 2. **Structure of a prokaryotic Argonaute with bound guide and target.** *a*, crystal structures of *Thermus thermophilus* Argonaute bound to a 10-mer DNA (*left*; DNA not shown to depict "apo"-Argonaute), a 21-mer guide DNA (*center*), or a 21-mer guide DNA with a 20-mer target RNA (*right*) illustrate Argonaute fold and function. The 5′-end of the guide DNA contacts the middle (*Mid*; *dark green*) and PIWI (*light green*) domains, and the 3′-end binds the N-terminal (*blue*) and PAZ (*purple*) domains. *b*, shown is a schematic representation of Argonaute domains and regions of interaction between the protein and guide strand (*dashed lines*). The *asterisk* denotes the location of slicer cleavage.

This intrinsic plasticity may explain how Argonaute proteins accommodate guide strands of various different lengths (3).

Mechanism of Target RNA Recognition

Upon engaging a target RNA, the nucleic acid-binding cleft in Argonaute opens to accommodate both guide and target strands (20). As illustrated in the ternary *Thermus* Argonaute crystal structure (Fig. 2*a*), bases 2–8 of the guide strand form a Watson-Crick-paired, A-form double helix with a complementary region of the target RNA. The remainder of the duplex is disordered, suggesting either that the complete duplex was not formed in the crystals or that the second half of the duplex remains mobile when bound to Argonaute. Both possibilities are consistent with the finding that the 3′-end of the guide RNA in human RISC pairs less efficiently with its target than the 5′-end (26, 27).

Bases 2–6 of the guide DNA are fully exposed and face outwards toward the bulk solvent in a near A-form conformation. This is a very important feature because bases 2–6 of the guide, termed the "seed region," are the most critical part of the guide for target recognition (25, 28). By protruding the seed region out toward the solvent, RISC may use these nucleotides as an initial probe for RNA targets as the complex traverses the cellular milieu. This would help explain the incredible efficiency with which RISC can locate its RNA targets. Kinetic analyses have shown that human RISC can find and cleave its target almost 10 times faster than the same small guide and target RNAs can anneal in free solution (26). Efficiency is also dramatically enhanced by the ability of RISC to loosely associate with single-stranded RNAs and perform a one-dimensional scan for matching target sites (26).

Consequences of Target Recognition

The core architecture of Argonaute and guide RNA allows RISC to efficiently locate specific targets within the vast pool of cellular RNAs. This capacity has been co-opted by evolution many times to generate distinct types of RISC that function in many different RNA-related processes (Fig. 3). In general, the consequence of recognition by RISC is down-regulation of the targeted gene. However, in principle, recognition could be evolved to function in any number of processes, including mRNA localization, alternative splicing, or even stimulation of gene expression. Indeed, several reports have implicated RISC in having a positive effect on target gene expression (29–31).

Slicing of Target RNAs—The simplest and best understood consequence of target recognition is mRNA hydrolysis, or slicing, which can break the reading frame of the encoded protein and promote target degradation by cellular exonucleases (1). The two requirements for target slicing are 1) a catalytically active Argonaute, *i.e.* a "slicer," and 2) a near-perfect sequence complementarity between guide and target RNAs, ensuring that only valid targets are cleaved (2, 32). Of the four Ago proteins in humans, only AGO2 is a catalytically active slicer (32).

The RNase activity of the Argonaute PIWI domain catalyzes the slicer cleavage reaction of target RNAs. This activity is dependent on divalent cations and is thought proceed via a two-metal mechanism, like many nucleases (2, 22). Essential for hydrolysis is a "DD(D/H)" catalytic triad, where the first and second positions are aspartic acid and the third position is aspartate or histidine (see Ref. 1 for a detailed list). The triad coordinates catalytic metal ions and positions a water molecule for nucleophilic attack of the phosphodiester backbone in the target RNA. Upon hydrolysis, the RNA is cleaved into two fragments, leaving a 5′-phosphate on one product and a 3′-hydroxyl on the other. Cleavage always occurs between the target nucleotides that pair with bases 10 and 11 of the guide strand (21, 33). At least *in vitro*, no other cellular factors beyond Argonaute and a guide RNA are required for the slicing reaction. In some cases, RISC can perform multiple rounds of target cleavage. In cases of multiple turnover, product release is generally held to be the rate-limiting step (2, 34, 35).

Translational Repression—The most prevalent mode of gene silencing by RISC in mammalian systems is the repression of translation guided by miRNAs. miRNAs are an abundant class of small regulatory RNAs found in plants and animals. miRNAs arise from endogenous transcripts that contain short double-

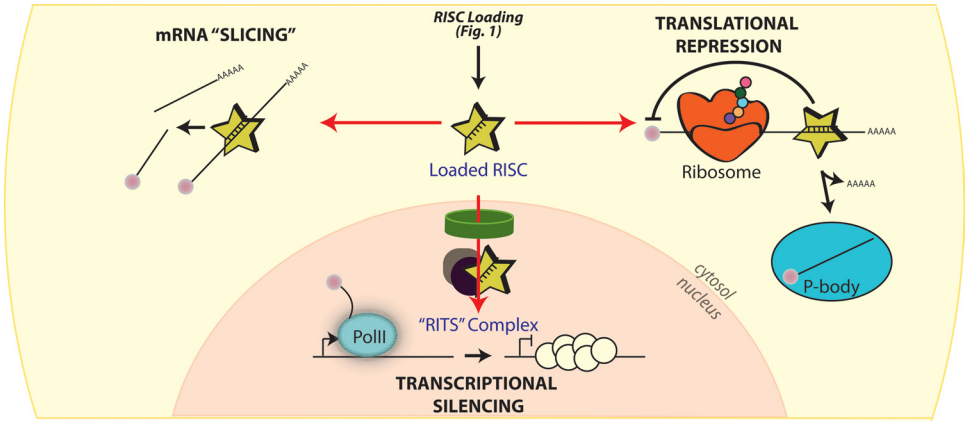

FIGURE 3. **RISC effector processes.** Once bound to its target RNA, RISC may down-regulate gene expression by one of several mechanisms, depending on the type of Argonaute and cellular context. In the cytoplasm, mRNA targets can be cleaved via RISC slicer activity or translationally repressed. In the nucleus, RISC can take the form of an RITS complex, which interacts with RNA polymerase II (*PolII*) and nascent RNA transcripts and directs chromatin remodeling to achieve epigenetic silencing.

stranded hairpin structures, which are processed and loaded into Ago proteins (Fig. 1). Unlike the slicing reaction, translational repression does not require extensive sequence complementarity between guide and target RNAs. As a general rule, only bases 2–7 of the guide RNA are required to match a target to initiate translational repression (25). Computational estimates suggest that each human miRNA targets between 100 and 200 messages, usually in the in 3′-untranslated region of the mRNA. Over 700 miRNAs are encoded in the human genome, and about one-third of all human genes are believed to be under the regulatory control of an miRNA (25).

There are several reported mechanisms by which RISCs can repress translation. Biochemically, translational repression is best understood in *Drosophila*, which possesses at least two distinct RISCs that each mediate repression by different mechanisms. The first mechanism involves inhibition of translation initiation. Specifically, RISC formed from *Drosophila* AGO2 can block protein-protein interactions between eukaryotic initiation factors 4E and 4G, which are required to form a competent pre-initiation complex on the target mRNA (36). On the other hand, *Drosophila* AGO1 represses translation by promoting target mRNA deadenylation and degradation. AGO1 RISC contains the protein GW182, which recruits the poly(A) deadenylation complex CCR4-NOT and the mRNA-decapping complex DCP1-DCP2 to target messages (37). GW182 is also involved in directing target mRNAs to cytoplasmic foci called P-bodies, which are translationally inactive structures that function as sites of mRNA storage and/or degradation (38). Mammalian RISCs employ similar mechanisms of translational repression (39); however, the relevant circumstances and exact mechanism(s) used by specific RISCs have yet to be determined.

Transcriptional Silencing and Formation of Heterochromatin—Beyond targeting mRNAs, some RISCs act directly on the genome. The best studied of these assemblies is the fission yeast RITS complex, which contains AGO1 with an associated siRNA, a protein called TAS3, and the chromodomain protein CHP1 (11). The RITS complex interrogates nascent transcripts as they are generated by RNA polymerase II in the nucleus. Upon target recognition, the complex recruits histone methyltransferases, which modify histones associated with the DNA locus, forming heterochromatin (40). The CHP1 subunit of the RITS complex specifically recognizes histone 3 proteins methylated at Lys^9, further reinforcing the association of the RITS complex with heterochromatin (41). The RITS complex also physically interacts with an RNA-directed RNA polymerase complex, which converts the targeted transcripts into dsRNA. Dicer then cleaves the dsRNA into new siRNAs, which can be loaded into new RITS complexes, thereby establishing a self-perpetuating silencing loop. Although the level of molecular detail is less well understood in other systems, plants and animals contain analogous systems for small RNA-guided formation of heterochromatin (42). In particular, the Piwi clade appears to function in transcriptional silencing and formation of heterochromatin (18).

DNA Elimination—The versatility of RISC function is perhaps best exemplified in the ciliate *Tetrahymena thermophila*. *Tetrahymena* has a complex genetic lifestyle involving two distinct nuclei in a single cell. The physically larger macronucleus is responsible for transcription during vegetative growth, whereas the micronucleus functions as a germ line. Following sexual conjugation, the paternal macronucleus is destroyed, and a new macronucleus is formed from mated micronuclei. During formation of the new macronucleus, any DNA sequences that were not present in the paternal macronucleus are eliminated, resulting in a loss of ~15% of the genetic material (43). DNA elimination requires the Piwi clade protein TWI1 (44). In the proposed model, the entire content of the newly formed micronucleus is transcribed into small RNAs called scnRNAs, which are then loaded into TWI1. The resulting RISCs then scan the entire genome of the old parental macronucleus. scnRNAs complementary to the old macronucleus are discarded, resulting in a filtered set of RISCs that target only DNA sequences new to the cell from sexual conjugation. These RISCs locate DNA sequences in the new macronucleus and tag them for elimination, most likely through histone methylation. The result is that new DNA sequences acquired through sexual conjugation are eliminated from the transcriptionally active macronucleus. This process likely functions as a defense mechanism against foreign parasitic DNA sequences and hinges on the ability of RISC to efficiently locate target sequences (43).

Future Prospects

RISC is an extremely versatile regulatory machine because it can be loaded with a guide RNA of any sequence and can be adapted to serve many distinct functions. A major challenge in the future will be to determine how many types of RISC actually function in living cells and the specific biochemical activities of each complex. Proteomic approaches have identified dozens of

Argonaute-associated proteins (5, 45), but relatively little is known about the biochemical role these proteins play in RISC function. Presently, the means by which binding partners interact with Argonaute and the extent to which these interactions are mutually exclusive are largely unknown.

Beyond understanding the molecular constitution of the various flavors of RISC, a clearer view of how post-translational modifications affect RISC activity will also be critical. Recent studies have found that human AGO2 is phosphorylated at Ser^{387} by a member of the mitogen-associated protein kinase (MAPK) cascade family. Phosphorylation promotes localization of AGO2 to P-bodies, indicating a role for regulating translational repression (46). Similarly, human AGO2 and AGO4 undergo prolyl hydroxylation specifically at Pro^{70}. This modification influences protein stability and may provide a scaffold for Argonaute-associated proteins to bind and impart new biochemical activities in a post-translationally regulated manner (47). Clearly, we are only beginning to understand the remarkable features of these multifarious regulatory machines.

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