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Point of View

The microRNA-argonaute complex

A platform for mRNA modulation

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With the cloning the *lin-4* gene in 1993, the possibility of an approximately 21-nucleotide RNA functioning as a regulatory molecule intrigued a relatively small number of scientists. This idea appeared to be a peculiarity of *C. elegans* as it was not until seven years later that the second, more conserved small RNA, *let-7* was cloned. A spate of papers in 2000 and 2001 revealed that the underlying properties of the *lin-4* and *let-7* genes were a common facet of animal genomes and the absolute number and potential of this new class of gene products requires us to integrate them with other aspects of gene expression and evolution.[1-3] A wealth of information has accumulated in the intervening years that outline, in general, how these small RNAs are expressed and processed into a functional form. Contemporaneous to these studies, experiments also identified a cadre of evolutionarily conserved proteins, the Argonautes (Agos) that directly associate with and are required for microRNA function. Computational and experimental methods have led the identification of many functional mRNA targets. In the last few years, a significant body of work has focused on resolving two key issues: How do microRNAs function in particular genetic contexts (i.e., as "molecular switches" or "fine-tuners" of gene expression) and secondly, what facet/s of mRNA metabolism do microRNAs modulate in their role(s) as a regulatory molecule? The primary objective here is not to comprehensively compare the competing models of microRNA function (reviewed in refs. 4–6) but to frame a potential solution to these two fundamental questions by suggesting that the core microRNA-Ribonucleic-Protein Complex (microRNP), composed of the microRNA and an Ago protein, functions as a highly modifiable scaffold that associates with specific mRNAs via the bound microRNA and facilitates the localized activity of a variety of accessory proteins. The resulting composite mechanism could account for the apparent complexities of measuring microRNA activity and furthermore, accommodate the broad levels of regulation observed in vivo.

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Mechanisms of microRNA Activity

A variety of mechanisms have been proposed to account for the dramatic effects microRNAs have on regulated transcripts. With a broad stroke, the mechanisms can be grouped into separate categories that modulate three possibly coupled facets of cytoplasmic mRNA metabolism: translation, mRNA localization and destabilization. Experimental assays designed to identify a single mechanism that explains the activity of microRNAs are complicated by the fact none of the highly tractable in vitro or in vivo systems leads to more than the two- to five-fold microRNA-dependent repression of heterologous reporters. This is in striking contrast to the almost complete repression of target gene expression afforded by some microRNAs in their endogenous context (*lsy-6*, *lin-4* etc.,).[7-13] Furthermore, particular microRNAs often differ in their overall effect on gene regulation; some microRNAs function as molecular switches within a genetic circuit while other microRNAs simply titrate or "fine tune" gene expression levels within a genetic pathway.[14,15] Nevertheless, all endogenous and reporter-based assays that measure microRNA-mediated repression clearly indicate that Ago proteins, via their ability to mediate protein-protein and protein-RNA interactions, are at the core of this form of regulation.

Three reports strongly indicate that part of microRNA-mediated repression may be accomplished by inhibiting the very earliest phases of mRNA translation. Kiriakidou et al. found that hAgo2 contains a stretch of amino acids with limited homology to a domain found in the translation initiation factor eIF4E required for mRNA cap-structure binding (Fig. 1A). Although the two proteins share little overall similarity, both proteins can bind the m^7G cap structure found on mRNAs. Mutations in the conserved aromatic residues of this inner Argonaute domain (termed the MC domain) ablate cap-structure binding by Ago2.[16] Because translational initiation proceeds through a complex interplay between the 5'-cap and 3' termini of the mRNA and this end-to-end linking is facilitated by eIF4E (the cap binding protein), eIF4G, and the poly A tail binding protein (PABP1), the authors propose a model in which hAgo2 competes with eIF4E for cap binding and reduces translation by limiting access of initiation factors to the 5' end of the transcript (Fig. 1A). Their experiments also demonstrate that mutations in the MC domain prevent Ago2 from binding the m^7G cap structure but do not inhibit all Ago2-related activities (mutant Ago2 retains its ability to bind microRNAs and RNAi "slicing" activites).

MC domain mutations dramatically reduce translational repression of experimentally targeted reporters.[16] Consistent with the competition model, Thermann and Hentze and Mathonnet et al. both developed cell free systems that recapitulate microRNA regulation and find that repression occurs at the initiation step of polyribosome formation. Thermann and Hentze find that transcripts repressed by mirR-2 quickly associate with an uncharacterized set of RNA-binding proteins that are molecularly distinct from proteins associated with translated mRNAs.[17] In the reconstituted reactions described by Mathonnett et al. microRNA activity leads to the rapid inhibition of mRNA translation and this repressive activity is sensitive to the levels of eIF4F present in extracts (an additional component of the cap-binding complex). As would be predicted from a competition-based mechanism, addition of excess eIF4F can overcome microRNA-mediated repression.[18] All three groups demonstrate that mRNAs utilize Internal Ribosomal Entry Sites (IRES, that bypass the requirement for most translational initiation factors) are resistant to microRNA repression. The in vitro system developed by Mathonnet also allowed the authors to demonstrate that transcripts containing an alternative cap structure (ApppG) were also refractory to microRNA-mediated repression, further indicating mRNA cap structure and translation initiation factors (the cap-binding complex) play a role in microRNA mediated repression.[18]

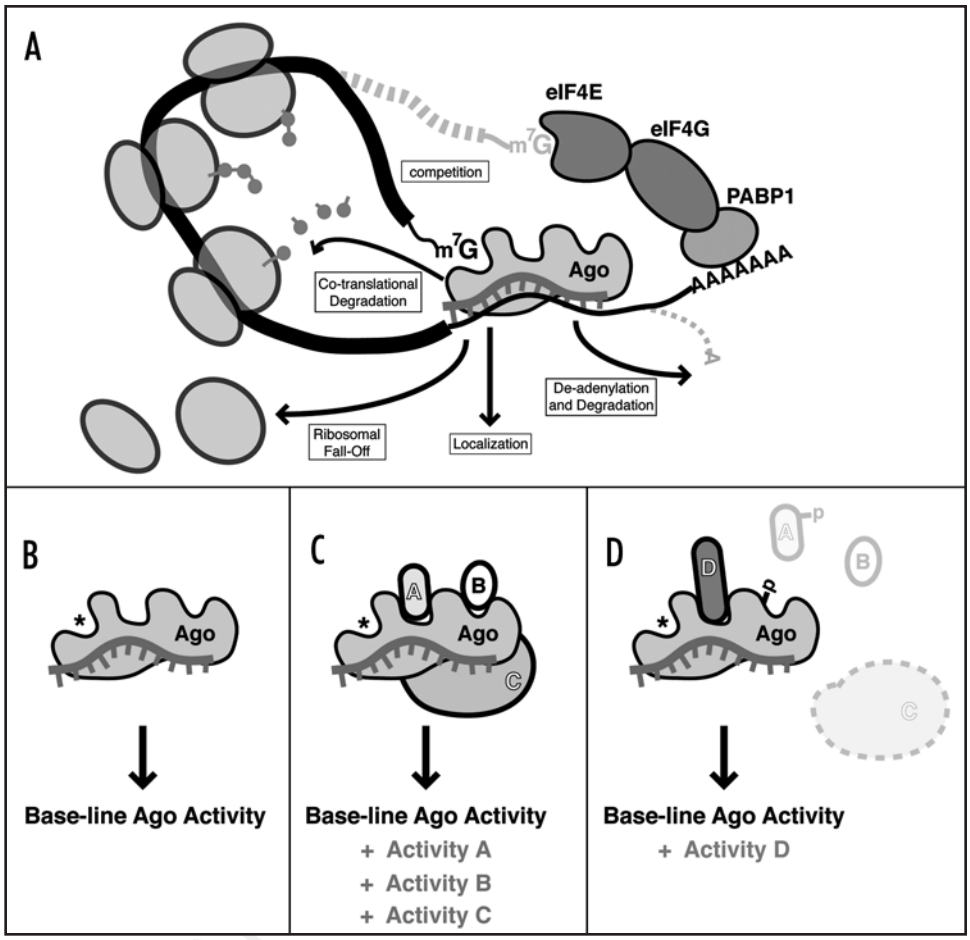

Figure 1. The microRNA/Argonaute complex functions as a molecular scaffold that modulates mRNA expression. (A) The miRNP complex recognizes target transcripts though interactions between the microRNA and partially complementary sequences in their 3'UTRs. As a consequence, the miRNP can modulate several aspects of mRNA metabolism. (B) The core components of the miRNP, the microRNA and Argonaute, can regulate mRNA targets through protein motifs that can directly interact with mRNA structures and translation initiation factors. (C) The core miRNP can interact with additional cofactors that mediate specific facets of mRNA regulation. The activity of each co-factor is independent of the other and are additive with regard to regard to the final levels of regulation on a target transcript. (D) Under specific circumstances or unique environments (i.e., serum starvation or within the germ line), the associations between the core miRNP and cofactors can be regulated by post-translational modification and or co-factor substitution. In this context, the resulting composite activity of a given microRNA can differ dramatically from its apparent activity in other conditions.

The elegant experiments described above suggest that at least some portion of microRNA-mediated repression occurs by the inhibition of translational initiation. Other lines of evidence suggest that microRNA-mediated regulation is more complicated. Initial biochemical analysis in *C. elegans* suggest that two *lin-4* mRNA targets, *lin-14* and *lin-28* are associated with polysomes both before (in early larval stage extracts) and during *lin-4* microRNA-mediated repression (in extracts of second larval stage animals).[19,20] This lead to the initial speculation that much of the *lin-4*-mediated repression occurs after translational initiation on transcripts that contain multiple, engaged ribosomes. The general nature of this proposed model is not limited to *C. elegans*. Multiple, independent studies using separate cultured cell lines also observed a polysomal association of transfected reporters under conditions in which endogenous or experimentally supplemented microRNAs were actively repressing these transcripts.[21-23] In each of the experimental systems outlined above the authors went to great lengths to show that the mobility of the repressed target transcripts in sucrose gradients was altered by a variety of conditions and drug treatments that disrupt engaged, 80S ribosomal subunits. In addition to these observations, a significant proportion many microRNAs are associated with the heavy fractions of polysomal gradients and their mobility in these gradients is limited by conditions that dissociate engaged ribosomes.[22,23] The mechanistic interpretations forwarded from these experiments included co-translational polypeptide destruction, peptidyl-transferase inhibition and ribosomal disassociation. Unfortunately, there is no direct experimental evidence to bolster any of these models as none of the components mediating any of these biochemical activities have been identified.

mRNA degradation has also emerged as a candidate mechanism for post-transcriptional repression mediated by microRNAs. Argonaute bound microRNAs effect mRNA turnover by at least three separate mechanisms: endonucleolytic cleavage and de-capping and deadenylation. In rare cases such as the evolutionally conserved regulation of mammalian HOXB8, miR-196 perfectly base pairs with

elements in the HOXB8 3'UTR causing the associated Argonaute protein to engage its "slicer" activity.[24,25] The HOXB8 transcript is cleaved between positions 10 and 11 relative to the 5' end of the microRNA as would happen with a siRNA mediated mechanism. "Sliced" HOXB8 transcripts are then degraded by the conventional RNA turnover components.[25]

In addition to the unconventional role of miR-196 in HOXB8 regulation, there are several clear reports that the activity of microRNAs lead to a dramatic enhancement of target mRNA turnover and the mechanism is fundamentally distinct from the endonucleolyticly-initiated cleavage mediated by the RNase H domains of the Ago proteins.[26-28] This observation has been used to identify and even validate several microRNA targets in vivo.[29,30] The clearest example of this type of microRNA-mediated regulation takes place during the transition from early, maternally supplied gene expression to that of zygotic transcribed mRNAs in the embryos of Zebrafish. Concurrent with the expression of early zygotic mRNA expression, miR-430 transcription is initiated.[31] Surprisingly, a significant percentage of maternally contributed transcripts (deposited during oogenesis) contain partially complementary miR-430 binding sites. miR-430 activity leads to the dramatic deadenylation of several maternally loaded target mRNAs and the presence of this microRNA correlates with the active degradation of hundreds of maternal transcripts.[31] Consistent with the specificity of this microRNA-mediated degradation, experimental ablation of miR-430 (via antisense oligonucleotides) leads to the inappropriate and continued expression of many maternal transcripts and the stabilization of specific, miR-430 regulated mRNAs.[31]

The generality of microRNA-mediated mRNA turnover can be verified in a variety of in vivo systems. In a tractable in vitro system this mode of repression can be shown to occur independently of translation.[32,33] Importantly, genetic ablation or knock-down (using RNA-interference) of a variety of known mRNA decay-pathway components [including decapping enzyme DCP1 and de-capping activators (e.g., RCK/p54 DCP1)] demonstrate that microRNA-mediated mRNA degradation occurs though this well characterized pathway.[26-28,32] However, the overall repressive activity contributed by this mechanism is hard to quantify since the microRNA-dependent decrease in target mRNA abundance is strongly dependent on the inherent stability and half-life of the targeted transcript as well as its relationship to a given microRNA. In addition, the location, number and context of all microRNA binding sites on a given UTR may dictate much of the overall turnover rate.[30,34] In a process that clearly requires cellular components related to mRNA turnover, microRNAs and their targets can also be compartmentally localized to several types of cytoplasmic foci including cytoplasmic processing bodies (P-bodies) or stress granules.[21,35-37] Although our current understanding of these subcellular compartments is only beginning to mature, two things are clear: (1) P-bodies and stress granules are not specific to microRNA-mediated forms of regulation, as most transcripts require the many of these components for normal mRNA turnover and (2) although several Argonaute proteins directly interact with some of the core components that define these structures, microRNAs can still inhibit the translation of their target transcripts in conditions that disrupt these complexes.[27,37,38]

…And microRNAs Can Function Positively

Two papers from the Steitz laboratory have fundamentally altered our understanding of the full potential of microRNA function and require a more generalized view of how these molecules operate in complex with Argonaute proteins. In contrast to the generally held assumption that microRNAs only mediate a repressive function on targeted mRNAs, Vasudevan and Steitz found that, under specific environmental conditions, microRNAs positively affect mRNA translation. Vasudevan et al. stumbled across this microRNA-mediated activity obliquely by characterizing a now classic model for mRNA destabilization mediated by AU-rich elements found in particular, post-transcriptionally regulated 3'UTRs (reviewed in ref. 39). Initially they observed that AU-rich elements of particular mRNAs [elements that under proliferative growth conditions lead to the elevated mRNA turnover (e.g., TNFα)] can stimulate translation under conditions of serum starvation.[40] Through affinity purification, Vasudevan and Steitz found that both Ago2 and a relative of a previously identified Ago2-interacting protein, the fragile-X-mental-retardation-related protein 1 (FXR1), both physically associate with translationally activated mRNAs and are required for the elevated translation in serum-starved environments.[40,41] Computational and candidate testing revealed that a particular microRNA, miR-396-3, is highly expressed in serum-starved conditions, is complementary to the AU-rich element found in TNFα and is required for translational activation under these conditions. Further characterization of other microRNAs, including *let-7* and even a synthetic microRNA miRcxcr-4, revealed that all microRNAs tested in these conditions could stimulate translation of mRNAs that contained cognate microRNA binding sites.[41] The Ago2 dependent repression or stimulation of translation could also be shown to function completely as a trans-acting protein complex by tethering a λN-tagged Ago2 protein to a mRNA transcript bearing the 5B-box RNA structural element to which the λN-protein binds with high affinity.[41]

Argonaute Proteins Provide a Molecular Platform for microRNA Activity

How can microRNAs function to regulate so many facets of mRNA metabolism and, in alternative environmental conditions, function in both a positive and negative manner? Furthermore, how can some microRNAs appear to function as "switches" and in other contexts merely "fine-tune" gene expression? The results outlined by Vasedevan et al. imply that the core components of the microRNA/Argonaute complex do not change and both the microRNA and Argonaute are required for both repressive and translational activating functions.[41] This suggests that Ago2 functions as a "scaffold" or "platform" that is targeted to particular mRNAs via specific base pairing by the bound microRNA. This scaffold can then be modulated by environmental, physiological or developmental inputs. In the case of translational activation, both the specific microRNA (or at least the ability to bind microRNAs) and a specific protein co-factor, FXR1, are required. Importantly, FXR1 does not appear to function in microRNA mediated repression and can not contribute to translational stimulation in the absence of a functional Argonaute.[41] One of the most intriguing ideas illuminated in these findings is that the modulation of Ago2 activity by FXR1 and serum starvation

is not a peculiarity of transcripts that contain AU-rich destabilizing elements but a variation of the standard mechanism of microRNA function.

One can imagine a solution for all competing mechanisms (Fig. 1A) proposed to answer the ultimate question of "how do microRNAs regulate target transcripts." Perhaps these are not competing models but are descriptions of many "true" mechanisms that occur in vivo. In each of the experimental systems used to "isolate" a particular mechanism, the experimenters have gone to great lengths to provide the proper controls and quantification to forward their particular hypothesis. Even the most convincing experimental systems typically only display between a two- to five-fold reduction in reporter expression which is typically articulated as the relative change in reporter expression compared to an additional, experimentally supplied, non-regulated reporter. Additionally, the calculation of the fold repression is complicated by the fact that mRNA transcripts typically give rise to more than one polypeptide in their lifetime and experimenters only measure the cumulative output of reporter expression that is distributed in a population of cells. In these experiments, culture conditions vary greatly and tissues or cells used in these assays are not always homogeneous in nature or physiological state (i.e., different cell cycle stage, serums conditions etc.). An alternative interpretation of a two- to five-fold level of repression would be that a given microRNA completely (i.e., irreversibly) represses just fifty percent (for two-fold reduction) to eighty percent (for five-fold) of the available targets in the population cells and the remaining portion of the reporter is not effected at all. Although there is a plethora of accumulating evidence that several endogenous microRNAs regulate their target transcripts within these physiological ranges (one- to two-fold), most of the microRNAs identified by forward genetic screens typically regulate the expression of there target transcripts in dramatic, almost "on" to "off," switch-like manner.[7-13] This leaves open the question of wheter microRNA regulation is much more effective for these "switch-like" targets, in vivo, or whether many mechanisms contribute to the total regulation of these targets.

One exception to the proceeding set of statements is the case of microRNA regulation of maternally loaded transcripts where much of the regulation can be accounted for by the single mechanism of active de-adenylation and subsequent degradation.[31] In this example, the regulation via miR-430 takes place in a very restricted and specialized environment. In this environment, one could imagine that the microRNP found in maturing embryos (which would be primarily loaded with miR-430 as it is one of the first zygotically transcribed microRNAs) could contain additional, germ-line specific protein components. These germ-line specific components would augment the normally modest levels of translational repression mediated by the streamlined, basal Argonaute/microRNA complex (Fig. 1B and C). A similar mechanism for augmenting basal Argonaute/microRNA complex activity could exist in serum starved cells where FXR1 association with the miRNP completely changes the apparent activity of this complex to one of translational stimulation. This could be accomplished by exchanging co-factors that normally repress mRNA expression for a wholly different set of components that lead to translational stimulation in serum starved conditions (Fig. 1C and D).

With this relatively simple hypothesis of a modular miRNP complex, one can begin to accommodate all of the experimental data that separately suggests a variety of disparate mechanisms for microRNA activity. In this hypothesis, the Argonaute and microRNA form the minimal inhibitory complex that leads to a basal level of microRNA-mediated repression (Fig. 1B). Repression would be mediated by the sequence specific recognition of the microRNA and the physical nature of the Argonaute protein and its ability to interact with fundamental and common structures (the m^7G cap structure) found in target transcripts and particular proteins (initiation factors and ribosomal recycling components) required for translation. The absolute level of inhibition mediated by this baseline activity may only represent the modest levels of regulation (one to two fold) seen in a variety of experimental assays.

Depending on the cellular context (i.e., cell type, developmental or physiological status etc.,) the core or minimal Argonaute/microRNA complex may associate with a variety of auxiliary proteins (Fig. 1C and D). Each of these auxiliary proteins could independently modulate the expression of a microRNA-targeted transcript. For instance, a given Argonaute/microRNA complex may contain co-factors that mediate critical interactions between P-body components, translational elongation machinery or even components of protein degradation machinery. In this scenario, the absolute level of regulation observed for a particular microRNA/target pair would be the composite of a variety of activities particular to the miRNP complex. Because the activity of a given microRNA would be a composite of these various physical interactions, each module could be individually regulated by post-translational modification of the co-factor itself or its interaction surface found on the Argonaute protein. This type of regulation would allow for exquisite control of the activity of a given microRNA or microRNAs. Additionally, due to the three dimensional structure of the basal Argonaute/microRNA complex, there would be sufficient structural information in the microRNA/target interaction to envision specificity factors. These specificity factors would recognize specific bulges or free sequence elements common to subsets of microRNA/target interactions. In this case, different targets of a given microRNA may display different levels of regulation depending on their ability to interact with one of these hypothetical specificity factors. Several reports have identified Argonaute interacting proteins that themselves have very specific phenotypes when mutated suggesting that they are not general components of the basal Argonaute/microRNA complex and therefore do not effect the function of all microRNAs.[42-44]

In summary, the idea that small RNAs could specifically regulate mRNA expression has already had a dramatic impact on modern biological research. It is hard to imagine how such relatively small snippets of RNA can lead to dramatic changes in target stability, translation and localization. One thin is clear: at the center of these interactions lie the Argonaute proteins. With the detailed characterization of additional microRNA/target interactions and the identification of the protein complexes that mediate their regulatory outcomes, one can only expect the list of Argonaute/microRNA cofactors to grow. For this reason alone, the core Argonaute/microRNA complex will remain the reference point of microRNA research.

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