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REVIEWS

Review

# Subcellular Heterogeneity of the microRNA Machinery

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**Different methods have recently been developed to understand the subcellular localization and role of microRNAs (miRNAs) as well as small RNAs associated with Argonaute (AGO) proteins. The heterogeneity of the protein complexes associated with miRNAs, along with their subcellular localization, provides clues into their biochemical mechanism of function. Subcellular diversity indicates that miRNAs localized to different cellular regions could have different functions, including transcriptional regulation on chromatin or post-transcriptional control, providing global regulation of gene expression by miRNAs. Herein, I review the current knowledge and most recent discoveries relating to the subcellular function of miRNAs and other AGO-associated small RNAs, revealing the emergence of a multitude of functions of the miRNA pathway to control different steps of the gene expression program(s).**

## Highlights

miRNAs are small noncoding RNAs that bind RNAs to primarily silence RNA and thus regulate gene expression.

miRNAs are localized in different cellular compartments in both the cytoplasm and nucleus, as well as in the extracellular milieu.

Subcellular localization of miRNAs is critical to facilitate proper protein and RNA interactions, and it would ultimately determine miRNA mode of action on target RNAs.

Recent data on nuclear miRNA function would challenge the current cytoplasmic-centric point of view of the miRNA-dependent mode of action.

Subcellular heterogeneity of the miRNA-dependent mechanism of gene expression control would ultimately adapt gene expression programs to normal and pathological events of physiology at different steps of the genetic information flow, from gene transcription to protein synthesis.

## The miRNA Pathway

MicroRNAs (miRNAs) are a class of small **noncoding RNAs** (**ncRNAs**; see Glossary) of approximately 22 nucleotides (nt) in length and are by far the most characterized regulatory ncRNAs. According to the last GENCODE release (v25), there are 1881 and 2202 different miRNAs in the human and mouse genomes, respectively (https://www.gencodegenes.org/). Together with other small ncRNAs, miRNAs are fundamental components of gene expression control that regulate all biological processes and may be used as biomarkers in human pathologies [1,2].

Briefly, primary miRNA transcripts are first processed by the DROSHA into an approximately 60 nt intermediate precursor called pre-miRNA [3]. Then, pre-miRNAs are cleaved by DICER into mature miRNAs, which are loaded into Argonaute proteins (AGO) [4,5] to form the miRNA-induced silencing complex (miRISC) [6].

Once loaded into the miRISC, miRNAs pair to sites within mRNAs to post-transcriptionally silence them [1]. In animals, miRNAs bind the 3′ untranslated region (UTR) mainly using a seed sequence of 6–8 nt in their 5′ end (from the second nt) to pair target sequences [7–9]. miRNAs silence mRNA translation by the deadenylation of the targets, which can be followed by decapping and often RNA degradation [10]. Recently, **mass spectrometry** analyses of AGO2-**immunoprecipitated** samples found that mammalian AGO complex contains hundreds of RNA-independent and RNA-dependent AGO-interactors [11,12]. Among them, some of the miRISC-associated proteins are known to play functional roles in either miRNA targeting or silencing mechanisms (Table 1). A key role is played by the three paralogue adaptor proteins, the trinucleotide repeat containing 6 (TNRC6A/B/C), which are required components for the miRNA-dependent silencing mechanism. TNRC6A/B/C have a single orthologue in flies, the glycine-tryptophan protein of 182 kDa (GW182), as well as two in nematodes, the ALG-1

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interacting protein (AIN1-2) [10,13,14]. TNRC6 proteins do not associate with other TNRC6 proteins [15]. Therefore, it is likely that each TNRC6 protein forms a complex with an individual AGO (four AGO proteins exist in mammals), resulting in the existence of 12 distinct TNRC6-AGO complexes. These complexes form molecular condensates within mammalian cells for the sequestration of miRNA targets and concentrates them with factors that mediate mRNA silencing [16,17] (Figure 1). In particular, the silencing domain of TNRC6A/B/C interferes with the translational machinery and accelerates mRNA degradation [18,19] by interacting with subunits of the deadenylase complexes, namely, PABP-dependent poly(A) nuclease 3 (PAN3), CCR4-NOT transcription complex, subunit 1 (CNOT1), and CNOT9 of PAN2–PAN3 and CCR4–NOT complexes, respectively [20–23]. Furthermore, TNRC6A/B/C also sequester the cytoplasmic polyadenylate-binding protein 1 (PABPC1) to promote translational blockade [24] and maximal deadenylation by destabilizing the eukaryotic translation initiation factor 4 G (EIF4G)-PABPC1 protective association on target mRNAs [25]. After poly(A) tail removal, degradation of the target mRNA body occurs by the decapping mRNA (DCP)1a-DCP2 5′-cap hydrolysis complex, the 5′-3′ exoribonuclease 1 (XRN1), and likely the exosome complex that degrades mRNAs in the 3′>5′ direction [10,17]. This biochemical cascade of miRNA-dependent silencing has been described in the cytoplasm.

Although more common in plants, few mammalian miRNAs were described to pair with target mRNAs with nearly complete sequence complementarity and cleave them through a slicing activity of AGO [1,26,27]. Furthermore, in particular cellular conditions, miRNAs have been found to enhance the translation of target mRNAs, such as in serum starvation [28]. Although the miRNA-dependent translational enhancement remains largely unclear, it appears to occur on targets lacking the cap and poly(A) tail [19]. The miRNA targeting and silencing mechanism have been extensively and recently reviewed in the literature [1,10,18].

miRNAs are very abundant molecules inside the cells and can be found virtually in all cellular compartments and in the extracellular milieu (Box 1). In this review, I provide an overview of the recent literature on the subcellular diversity of miRNA machinery in different cellular compartments/localizations, particularly with respect to their molecular mechanism(s) diversity, regulation, and pathophysiological functions in mammalian cells. I will attempt to integrate the many findings into a tentative framework that summarizes and rationalizes the global impact of the subcellular localization on miRNA-dependent gene expression.

## Cytoplasmic miRNAs

### Processing Bodies

The subcellular localization of miRNAs is critical to facilitate proper protein and RNA interactions and it may ultimately determine the miRNA mode of action on target mRNAs. Although an early study showed that AGO2 associates with the endoplasmic reticulum (ER) [29] (see below), subsequent studies have demonstrated that the cytoplasmic processing bodies (P-bodies) are the primary sites of miRNA activity in the cytoplasm [30] (Figure 2). In fact, immunofluorescence experiments conducted in mammalian cells have revealed that AGO2 has a punctate cytoplasmic localization when expressed as a tagged protein [31] or when using an anti-AGO antibody that recognizes the endogenous proteins [31,32]. The P-bodies, which are membrane-less organelles in the cytosol that vary in size from ~0.2 to 1 μm, are dynamically formed during the cell cycle upon extracellular cues [33,34]. Different RNA decay factors, such as DCP1a/2 and tristetraprolin (TTP) [31,32,35], which are involved in post-transcriptional processes are concentrated in P-bodies. These also include **nonsense-mediated mRNA decay, AU-rich element-mediated mRNA decay**, translational repression, and miRNA-mediated silencing [36].

## Glossary

**AU-rich element-mediated mRNA decay:** mRNA degradation controlled by cis-elements in the 3′UTR, called AU-rich elements (ARE), and ARE-binding proteins, including TTP, HuR, and others.
**Immunoprecipitation:** an affinity purification technique of specific antigens (proteins or protein complexes) using specific antibody (ies) that are immobilized on a solid support.
**Long noncoding RNA (lncRNA):** includes noncoding RNAs longer than 200 nt.
**Mass spectrometry:** an analytical technique utilized to identify and quantify biological material based on their chemical properties through the ionization of chemical species. Sorts the ions based on their mass-to-charge ratio.
**Noncoding RNA (ncRNA):** different groups of RNAs that are not translated into proteins, including rRNAs, tRNAs, snRNAs, lncRNAs, antisense-RNAs, miRNAs, and others.
**Nonsense-mediated mRNA decay:** a surveillance mechanism to mainly eliminate mRNAs that contain premature stop codons that would lead to aberrant dominant-negative proteins.
**Post-transcriptional control:** includes all mechanisms aimed at controlling gene expression at the RNA level, therefore between the transcription and the translation of the gene.
**RNA-binding protein:** about 8% of human genes encode proteins that bind RNA, and the list is still growing.
**SWI/SNF:** SWItch/Sucrose Non-Fermentable; a chromatin remodeling complex that promotes the transcription of a number of target genes.

Table 1. Main AGO Interactors in Mammals

| Gene name | RNA dependency of the interaction | Subcellular localization | Function on miRNA-dependent gene expression control | Refs |
|---|---|---|---|---|
| DICER | No | Cytoplasm, nucleus | miRNA processing | [11] |
| HSP70/90 | No | Cytoplasm | To load miRNA duplex into AGO | [5] |
| TNRC6A/B/C | No | P-body, ER, endosomes, exosome, chromatin, nucleoplasm | To sequester miRNA-target and recruit deadenylase complexes onto miRNA-target RNAs | [18], [78] |
| PAN3 | No | Cytoplasm, nucleoplasm, chromatin | To deadenylate miRNA targets | [10] |
| CNOT1 | No | Cytoplasm, nucleoplasm, chromatin | To deadenylate miRNA targets | [20–23] |
| CNOT9 | No | Cytoplasm, nucleoplasm, chromatin | To deadenylate miRNA targets | [20–23] |
| SRSF proteins | No | Nucleoplasm, chromatin | To regulate CD44 gene splicing | [86] |
| PABPC1 | Yes | Cytoplasm, nucleus | To promote translation. It is removed by miRISC on miRNA-target mRNAs. | [24] |
| DDX6 | Yes | Cytoplasm, nucleoplasm, chromatin, mitochondrion | To inhibit translation by removing the EIF4F complex from the miRNA target and promote its decapping by DCP1a/2 complex | [37] |
| MOV10 | Yes | Cytoplasm, nucleoplasm, chromatin, mitochondrion | To promote miRNA silencing in the P-bodies | [102] |
| TTP | Yes | Cytoplasm, nucleoplasm | To regulate miRNA targeting in selected mRNAs | [103] |
| HuR | Yes | Cytoplasm, nucleoplasm, chromatin, mitochondrion | To regulate miRNA targeting in selected mRNAs | [42] |
| hnRNP-Q | Yes | Cytoplasm, nucleoplasm, chromatin, ER | To inhibit miRNA silencing by competing with PABPC1 for poly(A) binding | [43] |
| SWI/SNF subunits | Unknown | Chromatin | To regulate transcription on the promoter | [87] |
| SFPQ/PSPC1/NONO | Yes | Nucleoplasm, paraspeckle | To promote miRNA targeting in selected mRNAs in the nucleoplasm | [11,12,83,91] |
| FUS | No | Nucleoplasm, paraspeckle, cytoplasm, chromatin | To promote miRNA silencing | [96] |

The miRNA pathway components TNRC6 proteins are enriched in these cytoplasmic foci [15]. In P-bodies, AGO also interacts with DDX6 (RCK-p54) [37], a DEAD-box RNA helicase that inhibits protein synthesis [38]. DDX6 uses its ATPase activity to release the EIF4F complex from the target mRNA, which would simultaneously repress translation and stimulate decapping [37] (Figure 1). Biochemically, interacting with CNOT1, DDX6 promotes the recruitment of the CCR4-NOT complex for mRNA degradation initiation [39]. In human cells, DDX6 inhibits HIF-1$\alpha$ (hypoxia-inducible factor 1$\alpha$) translation in the P-bodies, indicating a role for P-bodies in hypoxia stress [38].

Other cofactors are also required for miRNA-dependent silencing in P-bodies [40], as was demonstrated for the RNA helicase MOV10 that regulates a subset of miR-138 targets at the neuronal synapsis [41]. However, although miRNAs seem to be constitutively localized in the

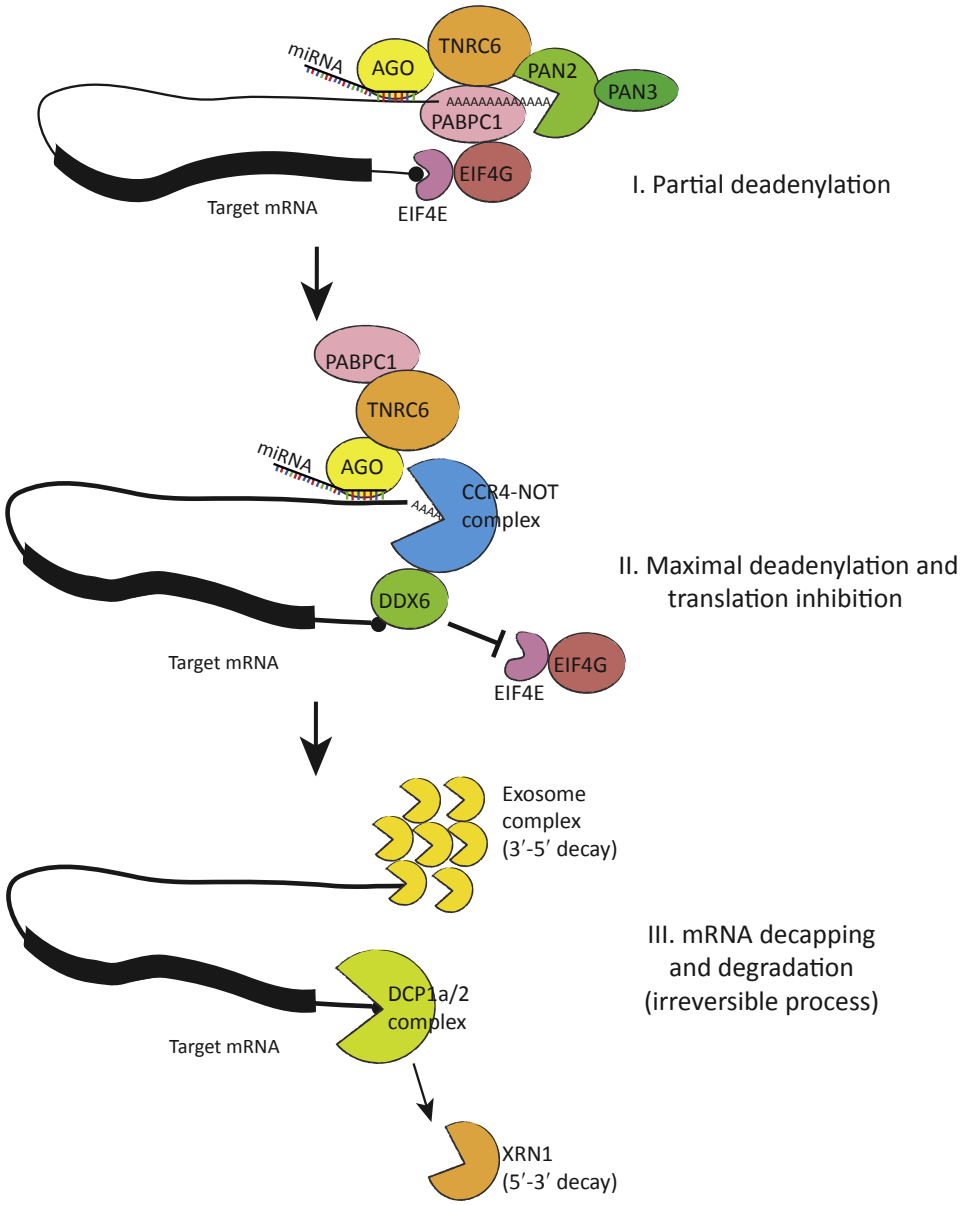



Figure 1. Overview of MicroRNA (miRNA)-Mediated Gene Silencing in Mammals. Mammalian miRNAs bound to Argonaute (AGO) proteins in the miRNA-induced silencing complex (miRISC) recognize their targets by base-pairing mainly on the 3' untranslated region (3'UTR) of the mRNA. AGO proteins interact with trinucleotide repeat containing 6 (TNRC6) proteins, which in turn promote the recruitment of the deadenylase PABP-dependent poly(A) nuclease 2 (PAN2)-PAN3 complex by direct interaction. This complex is thought to catalyze the first phase of the deadenylation reaction that is continued by the recruitment of the CCR4-NOT complex by direct interaction with TNRC6 proteins, whereas the eukaryotic translation initiation factor 4 E (EIF4E)/G complex is removed from the 5' cap of the target mRNA by the recruitment of the DDX6 protein. Then, the target mRNA can be decapped by the DCP1a/2 complex, which is the last step before the RNA degradation catalyzed by exoribonuclease 1 (XRN1) and the exosome complex for the 5' > 3' and 3' > 5' directions, respectively. PABPC1, Polyadenylate-binding protein 1.

Box 1. Extracellular miRNAs

Many reports indicate that small RNAs are detected in extracellular biological fluids, including blood plasma, urine, tears, breast milk, amniotic fluid, cerebrospinal fluid, saliva, and semen [104]. Extracellular small RNAs are very stable and can be used as biomarkers for the diagnosis or prognosis of different human pathologies [104,105] and function in cell-to-cell signaling via paracrine or endocrine routes [104]. Extracellular miRNAs can travel into apoptotic bodies, microvesicles, or membrane-free carriers, such as high-density lipoprotein (HDL), or are just associated with AGO [104] (Figure 2). Solely AGO-associated miRNA is by far the most represented form of extracellular miRNA [104]. Recent works reported that extracellular small RNAs associated with proteins, including the miRNA-AGO complex or other ribonucleoproteins, can regulate the innate immune response [2,106]. Interestingly, many groups have demonstrated that miRNAs travelling within apoptotic bodies, microvesicles, and HDL can be transferred to recipient cells, modulating gene expression and mediating functional effects [104].

Microvesicles, which include the exosomes, contain single-stranded miRNAs associated with AGO and TNRC6 proteins [59]. They originate from endosomal trafficking, which is thought to serve as mechanism to sort the miRNA content inside the exosome [58]. A large number of groups have independently demonstrated the functional role of exosomal miRNAs, in particular in cultured cells. Interestingly, it was recently shown that exosomal miRNAs, including miR-155, from adipose tissue macrophages of obese individuals cause glucose intolerance and insulin resistance by targeting adipocytes, muscle cells, and hepatocytes via paracrine and endocrine routes [107].

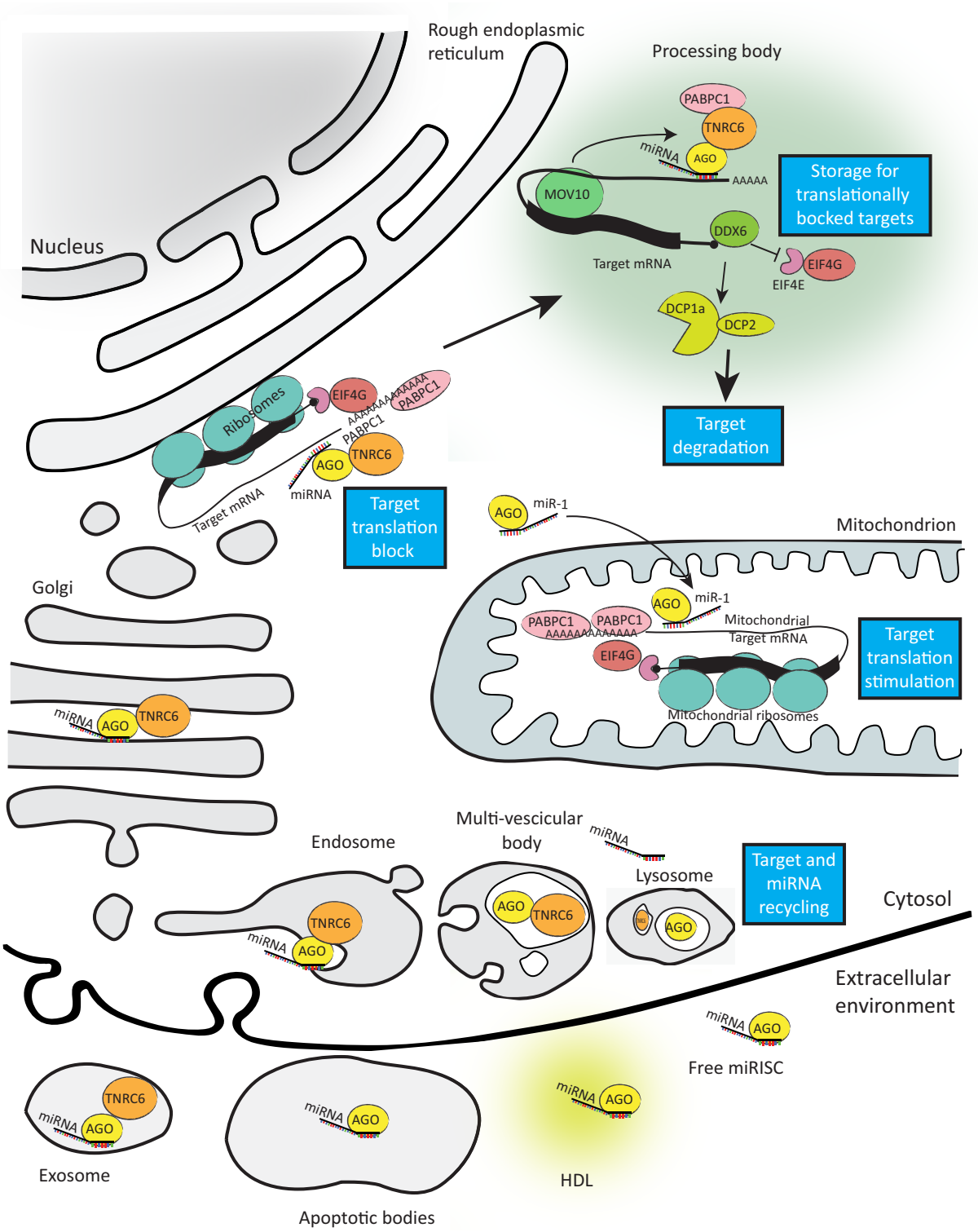
It is essential for future research to elucidate whether cell-type specific mechanisms exist for the intake of specific classes of circulating miRNAs.

P-bodies, different proteins have been demonstrated to control the P-bodies' localization of their targets. In fact, it was shown that HuR, by binding to the CAT-1 mRNA, releases the miR-122 targeting repression from P-bodies, which re-enters the polysomes [42]. Recently, it was also found that heterogeneous nuclear ribonucleoproteins (hnRNP)-Q inhibit miRNA-dependent silencing by acting as an antagonist with PABP for the binding to poly(A) to block deadenylation [43]. Interestingly, hnRNP-Q re-localizes to P-bodies upon propidium monoazide, thapsigargin, arsenite, and heat-shock treatments [44], suggesting that in particular stress conditions involved in different pathophysiological events, the global miRNA-dependent repression in the P-bodies can be reverted.

Together, translational inhibition and mRNA decay triggered by the miRNA pathway is associated with biochemical components enriched in P-bodies. The formation of P-bodies does not appear to be required for mRNA decay, but the miRNA silencing pathway is required for the integrity of P-bodies [36], suggesting that they are primarily a site of storage of translationally repressed mRNAs where degradation possibly occurs. In fact, in P-bodies, the destabilization of the EIF4F-cap complex by the action of pro-decapping factors would primarily repress translation [37]. For some mRNAs, this destabilization could follow the decapping reaction catalyzed by the DCP1a/2 complex, which is thought to be an irreversible process that targets the mRNA for degradation by the 5′ to 3′ exonuclease XRN1 [37]. However, since the 5′-3′ degradation factors and CCR4-NOT complex do not appear to be particularly concentrated in P-bodies but rather show a diffuse localization within the cytosol [45], one can deduce that the complete degradation of miRNA-target mRNAs does not occur in P-bodies. Nevertheless, it is unclear how miRNA-target mRNAs can be either stored and re-enter translation or initiate irreversible degradation. It has been hypothesized that the fate of mRNAs in the P-bodies may depend on their sequence and binding proteins [46], which, if it is experimentally confirmed, would open exciting new avenues for the full understanding of miRNA-dependent gene expression control.

### ER

In animals and plants, miRISC has been found to co-sediment with ribosomes and ER-bound polysomes (or rough ER) [47–50], indicating that the ER is a site of miRNA-mediated repression of gene expression (Figure 2). Although, conventionally the ER was reported to translate mRNAs

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encoding membrane-bound or secretory proteins, in many studies it was observed that transcripts encoding soluble proteins are associated with the ER or translated on membrane-bound polysomes [51]. It was found that miRNAs are associated with actively translating mRNAs, and rough ER acts as a site for miRNA-dependent translation repression [47,50,52,53]. In fact, the insertion of three let-7a binding sites in a luciferase reporter causes increased transcript levels in the ER-bound polysomal fraction in a let-7-dependent manner [47]. In plants, the altered meristem program1 (AMP1) may help miRNA targeting on mRNAs bound to ribosomes localized to the ER [52]. AMP1 is a membrane protein that localizes in rough ER and associates with AGO1 in the peripheral ER membrane in *Arabidopsis* [52]. Mutations in the AMP1 gene lead to multiple developmental defects in plants [52]. In humans, it was observed that AGO2 and other proteins involved in miRNA biogenesis and miRNA loading into AGO2 also associate with the rough ER [54].

Different cellular pathophysiological events impact on ER function, causing the accumulation of unfolded proteins inside the ER and leading to a specific condition referred to as 'ER stress'. In response to ER stress, cells launch the unfolded protein response (UPR), which is driven by three ER transmembrane proteins, namely, the protein kinase RNA-activated-like ER kinase (PERK), the activating transcription factor 6 (ATF6), and the inositol-requiring enzyme 1 (IRE1) [55]. UPR triggers apoptosis by translational control [55]. ER stress contributes to different human pathologies, including diabetes, neurodegenerations, and cardiovascular diseases. Interestingly, it was recently found that IRE1 RNase activation upon ER stress causes endonucleolytic cleavage of pre-miR-17, pre-miR-34a, pre-miR-96, and pre-miR-125b at sites distinct from DICER-dependent cleavage leading to their downregulation [56]. These miRNAs repress the translation of caspase-2 [56]. Therefore, during ER stress, IRE1 endonucleolytic activation antagonizes DICER activity on selected pre-miRNAs to de-repress the miRNA-dependent translation block on caspase-2 mRNA and entry into apoptosis [56]. These results indicate that the modulation of ER-localized miRNA-dependent silencing impacts the normal and pathological events of cell physiology.

Altogether, miRNA targeting of mRNAs in the rough ER would allow miRNA-mediated inhibition of translation and the early steps of miRNA-dependent gene expression programs. Given that the translation of mRNAs at the rough ER is more efficient than that in the cytosol [57], it can be speculated that miRNA-dependent translational silencing on the rough ER would permit a fast and more effective response to changes in environmental cues. Although, the functional and mechanistic connection between the rough ER and P-bodies is currently unknown, ER miRNA-target transcripts could be sequestered in P-bodies to prevent their translation and eventually initiate mRNA decay.

### Endosomal Trafficking Pathway

In a few independent studies, the miRISC, including the TNRC6 proteins, was observed to colocalize with the endosomal trafficking pathway, which includes the Golgi network, endosomes, multivesicular bodies, and lysosomes [58] (Figure 2).

Figure 2. Overview of MicroRNA (miRNA) Localization and the Mode of Action in Mammalian Cytoplasm. The first step of the miRNA-dependent mechanism occurs in the endoplasmic reticulum to inhibit the translation of mRNAs. Afterwards, miRNA-target mRNAs can be sequestered in the processing bodies (P-bodies) to prevent their translation and eventually to initiate mRNA decay by the DCP1a/2-dependent decapping reaction. It is thought that the full degradation of target mRNAs occurs in the cytosol. The endosomal trafficking pathway, including the Golgi apparatus, endosomes, multivesicular body, and lysosome regulates the turnover of the miRNA complex and their target mRNAs. Extracellular miRNAs are generated from the endosomes. Finally, AGO-associated miRNAs translocate to the mitochondrion to enhance the translation of mitochondrial mRNAs, as was shown for mito-miR-1 in myoblasts. AGO, Argonaute; EIF4E, eukaryotic translation initiation factor 4 E; HDL, high-density lipoprotein; PABPC1, polyadenylate-binding protein 1; TNRC6, trinucleotide repeat containing 6.

Some studies on this association also suggest that miRISC can load or disassemble at endosomes and multivesicular bodies for the recycling of AGO from miRNAs and target mRNAs [59,60], whereas the lysosome-dependent degradation of the content of multivesicular bodies may modulate the turnover of miRISC components [59,60] as well as the target mRNAs [61] (Figure 2). Interestingly, it was reported that in the neuronal dendrites the endosomal protein PICK1 (Protein Interacting with C Kinase – 1) interacts with AGO2 to inhibit the miRNA-mediated translational repression of key genes that regulate synaptic response to the induction of long-term depression [62,63]. The PICK1-AGO2 association is modulated by NMDA receptor stimulation [62]. In neurons PICK1 could interfere with the turnover of miRISC components and the target mRNAs to ultimately control the presynaptic translation of key proteins that control synaptic plasticity.

The endosomal trafficking pathway is mechanistically connected to autophagy; autophagosomes fuse with endosomes to be degraded in the lysosomes [64]. Autophagy is an intracellular process to modulate cellular homeostasis. Notably, it was found that the autophagy calcium-binding receptor and coiled-coil domain 2 (NDP52) co-immunoprecipitate with DICER, and the depletion of the core autophagy components autophagy related 5 (ATG5), ATG6, and ATG7 increases the levels of AGO and DICER proteins [65]. These data indicate that autophagy may ultimately control miRISC turnover and miRNA activity [58]. This mechanism may ensure homeostatic regulation by miRNA-dependent repression in human pathophysiology, including in cancer pathogenesis [66].

Because of the limited number of studies on the endosomal localization, more research is needed to fully elucidate the mechanism(s) played by the miRNA machinery in this compartment and its biochemical/biological relevance.

### Mitochondria

miRNAs that localize in the mitochondria are named mito-miRNAs, and can be either imported from the cytosol or internally originate from the mitochondrial genome [67]. It has been reported that small RNAs are imported into the mitochondria by two mitochondrial intermembrane proteins, the polynucleotide phosphorylase and the polyA polymerase [68], indicating that nuclear-encoded miRNAs can take the same route as nuclear-encoded proteins to localize and accumulate in the mitochondria. Selected miRNAs are found to be enriched in the mitochondria compared with the cytosol; for example, it was found that the nuclear-encoded miR-181c is twofold enriched in the mitochondria compared with the whole cellular extract of rat cardiomyocytes [69]. Mito-miR-181c was found to target mtCOX1 mRNA to block the translation [69]. Surprisingly, this result was challenged by another report finding that mitochondrial miR-1 coupled with AGO2 in differentiating myoblasts stimulates, rather than represses, the translation of mitochondrial transcripts (Figure 2), including COX1, to coordinate the myogenic program [70]. This opposite effect of mito-miR-1 compared with the cytoplasmic counterpart appears to be TNRC6-independent, since this protein is excluded from the mitochondria [70]. Whether this mechanistic disparity depends on the different sequence of the two miRNAs is an open question.

It is important to mention that the presence of controversial data on the mito-miRNAs may be caused by technical difficulties to isolate uncontaminated mitochondrial extracts from the cytoplasm or to precisely localize mito-miRNAs by immunofluorescence experiments [71]. These technical difficulties are also encountered to assess the nuclear and endosomal localization of miRNAs. Specific protocols to perform uncontaminated extractions of subcellular compartments/organelles or confocal microscopy have been recently developed to solve these

issues [72]. Unfortunately, in some publications the lack of important marker controls to assess the purity of the extracts or the immunofluorescence colocalization without confocal microscopy may lead to some controversy in the literature. In this regard, in the above-mentioned study of miR-1, the purity of mitochondrial preparations were shown by the absence of protein markers for the cytoplasm and the ER, and confocal technology was used for imaging AGO2 localization inside the mitochondria [70], whereas in the study of miR-181c the absence of 18S and 28S rRNAs was used [69].

In conclusion, mito-miRNAs could be involved in mitochondrial metabolism, mitochondrial oxidative phosphorylation, electron transport chain components, lipid metabolism, and cardiometabolic disorders [73]. Accelerating the investigations on mito-miRNAs and their mode of action may eventually lead to the design of novel mito-miRNA-based therapy for the treatment of mitochondria-related disorders.

## Nuclear miRNAs

The localization of miRNAs and AGO in the mammalian nucleus was first observed a decade after the discovery of miRNAs [74–77], indicating a nuclear role of the miRNA pathway. It was found that TNRC6A contains both a nuclear export signal and a nuclear localization signal, thus shuttling miRISC in and out the nucleus [78]. It was also reported that both TNRC6 and AGO proteins are imported into the nucleus by the importin-β pathway and Importin 8, respectively. [79,80].

In plants and yeast, AGO transcriptionally silences gene expression through epigenetic modifications that include RNA-directed DNA methylation (RdDM) [81] and histone methylation [81,82], respectively. However, such a nuclear function of metazoan miRNAs has not been widely appreciated, mainly because the potential mechanism involved has remained largely unknown for a long time.

Mass spectrometry analyses of the protein complexes associated with nuclear AGO and TNRC6 proteins in human cells revealed the presence of several proteins involved in different biochemical pathways [83,84]. Notably, nuclear AGO2 interacts with TNRC6 proteins, which directly associate with several enzymes and cofactors, including components of the CCR4-NOT complex [85], as well as some transcriptional and DNA repair regulators [86,87]. The conserved interaction between TNRC6s and the CCR4-NOT complex between the cytoplasm and nucleus would indicate that the deadenylation activity of miRNA targets could also occur in the nucleus. However, due to its modular structure, the nuclear CCR4-NOT complex is also involved in broader nuclear activities in addition to its deadenylation function, including protein ubiquitination [88] and as a negative regulator of transcription [89]. Thus, miRISC could potentially take part in these mechanisms.

Since nuclear AGO does not interact with HSP90, which is a cofactor involved in miRNA-loading activity into AGO, it is suggested that nuclear miRNAs are not locally produced but instead imported from the cytoplasm along with AGO proteins [72]. Nuclear miRNAs can be found to either be associated with chromatin or in the nucleoplasm (Figure 3).

### Chromatin

AGO proteins interacting with chromatin can act in conjunction with small RNAs to regulate either transcription or splicing. Two studies determined the AGO interactome on the chromatin of human cells. The first study, published in 2012, identified chromatin modifiers and SRSF splicing factors associated with AGO proteins by mass spectrometry analysis using an anti-flag antibody to immunoprecipitate flag-tagged AGO1 or AGO2 on the chromatin [86]. It was

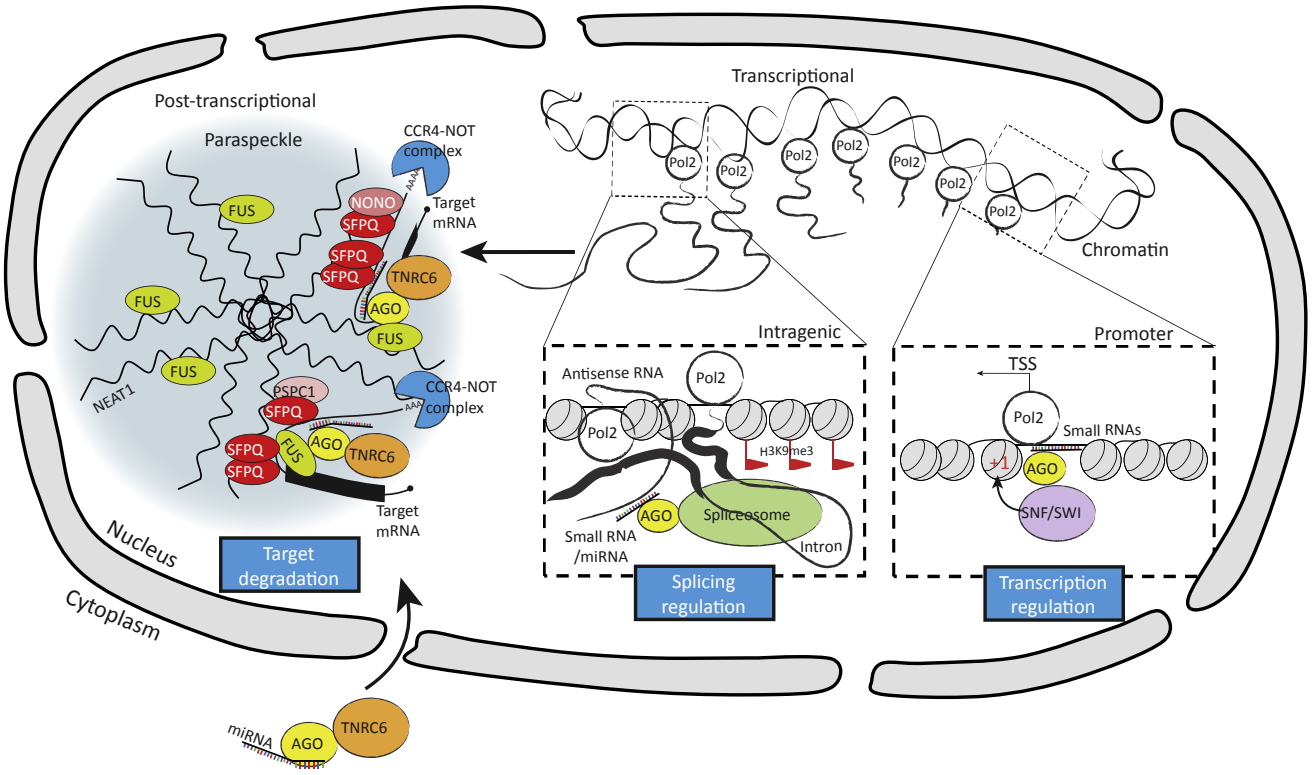


Figure 3. Overview of MicroRNA (miRNA) Localization and the Mode of Action in the Mammalian Nucleus. Nuclear miRNAs mainly localize in the nucleoplasm to target mRNAs and promote RNA degradation by a CCR4-NOT complex-dependent mechanism. miRNA targeting of nuclear mRNAs is promoted by the RNA-binding proteins SFPQ, NONO, PSPC1, and FUS, which belong to the paraspeckle. The paraspeckle is a nucleoplasmic compartment generated by the long noncoding RNA NEAT1. I speculate that the paraspeckle may function as a nuclear site for SFPQ/PSPC1/NONO/FUS-dependent miRNA target repression by CCR4-NOT complex degradation. In addition to their nucleoplasmic function, Argonaute (AGO) proteins associate with small RNAs on chromatin to control either the transcription or splicing of nascent RNAs. TNRC6, Trinucleotide repeat containing 6; TSS, transcription start site.

observed that AGO1 and AGO2 regulate the pace of the RNA polymerase 2 elongation to ultimately modulate alternative splicing of CD44 pre-mRNA. AGO1 and AGO2 on nascent RNAs are associated with small RNAs, including miRNAs, and correlate to histone H3 lysine 9 methylation on the CD44 locus (Figure 3). In the second study, using mass spectrometry analysis of endogenous immunoprecipitated AGO2, **SWI/SNF** was found to associate with AGO2 [87]. It was found that the complex made by AGO2 and SWI/SNF proteins associates with DICER-dependent small RNAs, which map to the promoter nearby the transcription starting site, to possibly modulate the first nucleosome occupancy and thus the transcription activity [87] (Figure 3). It is noteworthy that the interaction between AGO2 and SWI/SNF proteins was never demonstrated on the chromatin, leaving open the possibility that this association may be functional in the nucleoplasm.

## Nucleoplasm

Importantly, the majority of nuclear miRISC localizes in the nucleoplasm, whereas just a small part localizes on the chromatin [12]. Nucleoplasmic miRNAs possess a post-transcriptional silencing activity [78]. It was found that nuclear miR-9 targets the nucleoplasmic long noncoding RNA (lncRNA) MALAT1 by an AGO2-dependent mechanism, proving a functional and

biochemical link between miRNAs and lncRNAs that occurs in the nucleoplasm [90]. Different groups observed that miRISC interacts with the nuclear *Drosophila* behavior/human splicing (DBHS) proteins [11,12,83,91], namely, SFPQ, PSPC1, and NONO. In particular, it was recently observed that SFPQ mediates the RNA-dependent interaction between the DBHS proteins and the miRISC on mRNAs that contain long 3′UTR [12]. SFPQ would likely modulate the secondary structure of target 3′UTR, ultimately favoring the binding of miRNAs on a specific sequence located within 500 nt from SFPQ-binding sites and therefore promoting a miRNA-dependent degradation of target mRNAs (Figure 3). Despite its nucleoplasmic localization, SFPQ regulates miRNA-binding activity in both the nucleoplasm and cytoplasm, suggesting that it promotes a nucleoplasmic commitment of mRNAs to globally control miRNA targeting. These findings would challenge the current cytoplasmic-centric point of view of miRNA-dependent degradation of target RNAs. Functionally, it was found that SFPQ regulates the let-7a-dependent differentiation program in stem cells by regulating the let-7a targeting of crucial mRNAs, including Lin-28 homolog A (LIN28A), insulin like growth factor 2 (Igf2bp1), and hypermethylated cancer 2 (Hic2), most likely by a nuclear CCR4-NOT complex-dependent degradation mechanism [85].

Both SFPQ and NONO were recently found to promote miRNA biogenesis together with the lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) [92]. Importantly, SFPQ, NONO, and NEAT1 are essential components of the paraspeckle, which is a nucleoplasmic compartment of approximately 0.2–1 μm in size. Together, these observations raised the exciting possibility that the paraspeckle would function as a nuclear body that couples miRNA biogenesis and targeting to initiate miRNA silencing of a subset of mRNAs containing long 3′UTRs via a CCR4-NOT-dependent degradation, which can be differentially regulated upon changes of microenvironmental cues [93]. Interestingly, FUS, which is a nuclear **RNA-binding protein** involved in miRNA biogenesis [94] and is also an essential protein of the paraspeckle [95], was recently found to regulate miRNA-mediated gene silencing [96]. Particularly, in cells expressing a truncated mutant form of FUS, which is present in some patients with amyotrophic lateral sclerosis and lacks the nuclear localization signal, the miRNA-dependent silencing is impaired, suggesting that FUS may promote miRNA targeting and silencing in the nucleoplasm, and in particular in the paraspeckle. Together, these data indicate that the paraspeckle may serve as a nuclear site for **post-transcriptional control** of the miRNA-dependent gene control. The miRNA pathway could impact the different functions of the paraspeckle [97], including nuclear retention of mRNAs [98], transcriptional regulation [99], cancer pathogenesis [100], and viral infection [101].

## Concluding Remarks

More than two decades have passed since the discovery of miRNAs, and yet the mechanism by which they regulate gene repression is still a matter of contention, in particular in the field of subcellular functional diversity. Growing evidence suggests that the mechanism of miRNA-dependent gene silencing is more complicated than can be explained by a single model. By studying how miRNAs biochemically regulate their direct targets, we are learning more about how cells adapt gene expression programs to normal and pathological events of physiology at different levels of the genetic flow of information and in all cellular compartments. Subcellular localization of miRNAs may be necessary to regulate specific processes that occur inside the organelles or subcellular structures, such as mitochondrial metabolism for mito-miRNAs or synaptic plasticity for endosomal miRNAs. miRNAs that localize in the ER and P-bodies, which can block the translation and store target mRNAs, can fast adapt gene expression program(s) to temporary cellular conditions in pathophysiological events, such as normal homeostasis versus stress response during, for example, hypoxia stress response. Finally, nuclear miRNAs,

## Outstanding Questions

Although the miRNA complex and its localization have been extensively studied in the past decades many questions remain unanswered.

The first question to be solved regards the regulation of miRISC subcellular localization; how are miRISCs sorted within the cell and where do they encounter their targets?

Next, what is the specific contribution of each miRISC localized in different subcellular compartments on target mRNA control, and what is its quantitative impact on gene expression programs and cellular response/physiology?

The nuclear localization and function of miRISC have recently emerged as an additional layer of complexity for miRNA-regulated gene expression programs. Nuclear miRNAs have been shown to be capable of being both transcriptional and post-transcriptional/decay regulators of gene expression; however, their biochemical mechanisms have yet to be totally disclosed. It would therefore be important to investigate the molecular partners of nuclear miRISC and their effects on target mRNAs; for example, are decapping and decay reactions mediated by nuclear miRNA binding to target mRNAs? What are the enzymes involved in the nuclear miRNA-dependent degradation?

Is the paraspeckle a major site for gene silencing by nuclear miRNAs? Could the paraspeckle organization enhance the nuclear miRNA targeting/silencing of target RNAs, by, for instance, a phase separation mechanism?

which silence target genes both transcriptionally and post-transcriptionally, could change more profoundly the phenotype of the cell and therefore be part of dramatic cellular processes, such as cell differentiation or transformation. With many questions remaining about miRNA biology (see Outstanding Questions) I am confident that the field will continue growing, providing new data to fully understand the complex code governing gene expression control.

## Acknowledgments

I am grateful to Drs Valerie Grandjean and Emanuela Repetto for their helpful reading of this manuscript and apologize to scientists whose work I could not cite due to space limitations. This work was supported by the ANR through the 'Investments for the Future' # ANR-11-LABX-0028-01 (LABEX SIGNALIFE), FRM (grant #DEQ20140329551), Agence de la Biomédecine (AMP, Diagnostic prénatal et diagnostic génétique), and the Société Française de Nutrition (Prix de Recherche 2017).

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