MicroRNA-mediated silencing of natural UTRs in *C.elegans* embryos is prevalent and requires miRISC collaboration

By

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ABSTRACT

MicroRNAs (miRNAs) are small RNAs that play a pivotal role in post-transcriptional gene regulation. These regulatory RNAs associate with the Argonaute proteins to form the miRNA-induced silencing complexes (miRISCs). In metazoans, miRISCs typically target mRNAs by imperfectly binding to complementary sites in 3' untranslated regions (3'UTRs), thereby affecting the translation of the targets, and/or reducing their stability. Despite the significant roles miRNAs play in various biological processes, the mechanistic details of how they regulate gene expression remain unclear. Using a *C. elegans* embryonic *in vitro* system, we focus on the mechanism for miRNA mode of action, and the significance of the poly(A) tail in miRNA-mediated silencing during development. We show that our miRNA luciferase reporters underwent deadenylation starting at 20 minutes of incubation of the RNA with *C. elegans* extract, and this process is dependent on the Argonautes involved in the miRNA pathway, ALG-1 and ALG-2. We also detect the presence of an RNA decay intermediate within two hours of target RNA-extract incubation. The appearance of this intermediate is independent of the m^7GTP cap, indicating a 3'→5' decay pathway occurring in coordination or independently of miRNA-mediated deadenylation. Furthermore, we present here our screen for endogenous targets of the maternal *miR-35-42* family, a miRNA family abundantly expressed in the embryo and essential for embryogenesis, via deadenylation assays. From our screen, we identified the tolloid/BMP-1 family member, *toh-1*, as a deadenylated target of *miR-35-42*. The pro-apoptotic *egl-1* was also identified as a target of *miR-35-42,* as well as the zygotically expressed *miR-58*. Our findings demonstrate that more than half of the predicted natural UTRs were deadenylated in a miRNA-dependent manner. We also show that a minimum spacing is required for miRISCs to efficiently silence their targets, and we illustrate that at least two separate miRISC-binding sites are required to drive deadenylation. Our findings provide insights into the biochemistry of miRNA action, the prominent role played by miRNA-mediated deadenylation, and the biological implications of maternal and zygotic miRNAs in development.

RÉSUMÉ

Les microARNs (miARNs) sont des petits ARNs qui jouent un rôle important dans la régulation post-transcriptionnelle des gènes. Ces ARNs régulateurs s'associent à des protéines, nommées les Argonautes, afin de former un complexe de répression induit par les miARNs (miRISCs). Chez les métazoaires, les miRISCs ciblent l'expression des gènes par une hybridation imparfaite avec la région non-codante en 3' (3'UTR) de l'ARN messager (ARNm) ciblé, ce qui a pour effet d'affecter la traduction des ARNm, et/ou de réduire leur stabilité. Malgré le fait que les miARNs jouent plusieurs rôles significatifs dans divers processus biologiques, leur mécanisme de contrôle de régulation génique demeure incompris. En utilisant un système *in vitro* chez les embryons de *C. elegans*, on se concentre sur le mécanisme d'action des miARNs et sur l'importance de la queue de poly(A) dans la répression des ARNm par le biais de miARNs pendant le développement. Nos résultats démontrent que suite à l'incubation de l'ARN avec l'extrait de *C. elegans,* nos gènes rapporteurs de luciférase-miARN ont commencé à être déadénylés après 20 minutes. Ce procédé est dépendant des Argonautes ALG-1 et ALG-2. On a aussi détecté la présence d'un deuxième ARN intermédiaire plus court après deux heures d'incubation de l'ARNm ciblé avec l'extrait. L'apparition de cet intermédiaire est indépendante du cap m^7GTP, indiquant une voie de dégradation 3'→5'. On présente également un essai de déadénylation pour examiner les ARNm endogènes ciblés par la famille des miARNs maternelles, *miR-35-42*. Cette famille de miARNs est exprimée abondamment dans l'embryon et est essentielle pour l'embryogenèse. On a identifié un membre de la famille tolloid/BMP-1, *toh-1*, comme un ARNm ciblé et déadénylé. Le pro-apoptotique *egl-1* a aussi été identifié comme un ARNm ciblé de la famille *miR-35-42* ainsi que de *miR-58*, un miARN exprimé zygotiquement. Nos résultats démontrent que plus de la moitié des 3'UTR endogènes de nos ARNm qui étaient des cibles candidates sont déadénylés de manière dépendante des miARNs. On montre aussi qu'il y a une distance minimum requise pour que la répression par les miRISCs soit efficace et qu'au moins deux sites miRISC sont nécessaires pour permettre le déadénylation. Nos résultats donnent un aperçu du mode d'action biochimique des miARNs, du rôle important joué par le biais de

miARNs sur la déadénylation et des implications biologiques des miARNs maternels et zygotiques au niveau du développement.

ABBREVIATIONS

2'-*O*-Me	2'-*O*-methyl
A-cap	ApppG cap analog
Ad	adult
AGO	Argonaute
AIN	ALG-1 INteracting protein
ALG	Argonaute-Like Gene
ARCA	Anti-Reverse Cap Analog
ATP	adenosine triphosphate
BH3	Bcl-2 homology region 3
BMP-1	bone morphogenic protein-1
bp	base pair
C-	negative control
cDNA	complementary DNA
C. elegans	*Caenorhabditis elegans*
CGH-1	conserved germline helicase
chr.	chromosome
Ci	Curie
CLL	chronic lymphocytic leukemia
CTP	cytosine triphosphate
DCP	decapping protein
DCR	Dicer
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
ee	early embryo
egl	egg-laying
eIF	eukaryotic initiation factor
fem-1	feminization of XO and XX animals
F1	first generation
FL	Firefly luciferase
fwd	forward
glp-4	abnormal germline proliferation-4
GFP	green fluorescent protein
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGA2	high mobility group A2
IPTG	isopropyl β-D-1-thiogalactopyranoside
KCl	potassium chloride
KOAc	potassium acetate
KOH	potassium hydroxide
L1	larval stage 1
L2	larval stage 2
L4	larval stage 4

le	late embryo
let	lethal
Limk1	Lim-domain-containing protein kinase 1
lin	lineage
LNA	locked nucleic acid
m^7GTP	7-methyl guanosine triphosphate
mCi	milli Curie
me	middle embryo
$Mg(OAc)_2$	magnesium acetate
miRISC	microRNA-induced silencing complex
miRNA	microRNA
miRNP	miRNA-ribonucleoprotein
mRNA	messenger RNA
MZT	maternal-to-zygotic transition
NaCl	sodium chloride
NGM	nematode growth medium
ORF	open reading frame
p(A)	poly(A)
PAGE	polyacrylamide gel electrophoresis
PAP	poly(A) polymerase
P-bodies	processing bodies
pre-mRNA	precursor mRNA
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTMs	post-translational modifications
Q-PCR	real-time polymerase chain reaction
rev	reverse
RNA	ribonucleic acid
RL	Renilla Luciferase
RNAi	RNA interference
RNase	ribonuclease
RNasin	RNase inhibitor
rRNA	ribosomal RNA
RT-PCR	reverse-transcription polymerase chain reaction
spn	spindle
SSC	saline sodium citrate
TGF	transforming growth factor
toh	tollish
tRNA	transfer RNA
UTP	uracil triphosphate
UTR	untranslated region
XRN-1	XRN (mouse/*Saccharomyces cerevisiae*) ribonuclease related family member
Zn	Zinc

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CONTRIBUTION OF THE AUTHORS

This thesis is presented in the traditional format according to the McGill University guidelines for Thesis Preparation (http://www.mcgill.ca/gps2/students/thesis/programs/guidelines/).

The work presented in this thesis was entirely my own, but I would like to acknowledge the following individuals who have made contributions to the data presented in Chapter 3 of this thesis:

- Caroline Thivierge performed the microRNA expression profiling by northern blots and qRT-PCR , and the 2'-*O*-Me pulldown experiments (Fig. 5 and Fig. 6)
- Mathieu Flamand performed the natural 3'UTR cloning, as well as the cloning and sequencing of the deadenylated *miR-35* targeted reporters (Fig. 9D).
- Dr. Marc R. Fabian conducted the RT-PCR amplification of *miR-35* targeted reporters.

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CHAPTER 1: INTRODUCTION

1.1 Overview of microRNAs

MicroRNAs (miRNAs) represent a class of small RNA molecules that regulate gene expression post-transcriptionally. These small regulatory RNAs are implicated in a wide range of biological processes that include development, cell proliferation and differentiation, apoptosis, neurobiological processes, and metabolism. Regulation by miRNAs is evolutionarily conserved. miRNAs have been identified in many organisms and the number of miRNAs encoded by the genome can range from a hundred (in organisms including *Drosophila melanogaster* (*D. melanogaster*) and *Caenorhabditis elegans* (*C. elegans*) to at least a thousand (in humans)[1-3].

In recent years, extensive research on miRNAs has provided insights into our understanding of their mechanisms and biological functions. These small regulatory RNAs are predicted to modify the expression of 30% of all protein-coding genes[4]. By way of RNA-RNA interactions, miRNAs can affect both translation and stability of their mRNA targets. A number of predictive algorithms are available to identify target mRNAs for miRNAs and vice versa. To date, few targets have been validated experimentally. Despite being an active research field, the biological function of miRNAs and their mechanisms of action are not well understood.

1.2 Discovery

The very first miRNAs discovered and insights into the mechanism for miRNA action originated from the nematode *C. elegans*[5-7]. In a search for genes involved in *C. elegans* developmental transitions, Ambros and Horvitz noticed mutants in the *lin-4* gene reiterated the first larval stage (L1)-specific developmental events at later larval stages[8]. *lin-4* was identified as a repressor of *lin-14*, a heterochronic gene which encodes for a transcription factor crucial for the completion of L1[5, 9]. Unexpectedly, *lin-4* was found to encode a small RNA of 22 nucleotides rather than a protein. Analysis of *lin-4* RNA and *lin-14* mRNA revealed seven *lin-4* binding sites within *lin-14* 3' untranslated region

(UTR). The interaction between *lin-4-lin-14* involves imperfect base pairing, suggesting *lin-4* RNA negatively regulates *lin-14* mRNA through a RNA-RNA interaction (Fig. 1A)[10, 11]. Another miRNA, *let-7*, was identified shortly thereafter to regulate the expression of LIN-41, a protein required for the fourth and final larval stage (L4) to adult transition. Similarly to *lin-4*, loss of *let-7* miRNAs also results in heterochronic defects, such as the reiteration of earlier cell-fate lineage. The ability of *lin-4* and *let-7* RNAs to affect the protein synthesis by base-pairing to the 3' untranslated region (3'UTR) of their target mRNAs and exert a temporal regulation led miRNAs to be initially termed "short temporal RNAs" involved in translational repression[5, 7].

The discovery of *lin-4* and *let-7* as founding members of a new class of RNA with a regulatory function led to the search for other similar regulatory RNAs. In 2001, almost a decade after the initial discovery of *lin-4* and *let-7*, this class of small RNAs extended to *D. melanogaster* and humans, and many more short regulatory RNAs were also reported in *C. elegans*[12-14]. The diversity and abundance of these small RNAs suggest that miRNAs are evolutionarily related and play a broad role in animals. The discovery of miRNAs sparked the search for their roles, their targets, and the mechanism behind RNA regulation.

1.3 Biological functions

Since their discovery in *C. elegans*, miRNAs have emerged as more than regulators of developmental timing. Several examples will now outline the significance of miRNAs in different cellular and developmental contexts.

1.3.1 miRNAs and animal development

Numerous studies have shown that miRNAs are overall essential for animal development. Loss of the miRNA-processing enzyme, Dicer, abolishes miRNA production and results in developmental arrest. In zebrafish, loss of Dicer results in a block in development and growth arrest after 10 days[15]. In mice, several studies have reported early embryonic lethality after 7.5 to 14.5 days of development upon loss of Dicer[16, 17]. Loss of specific

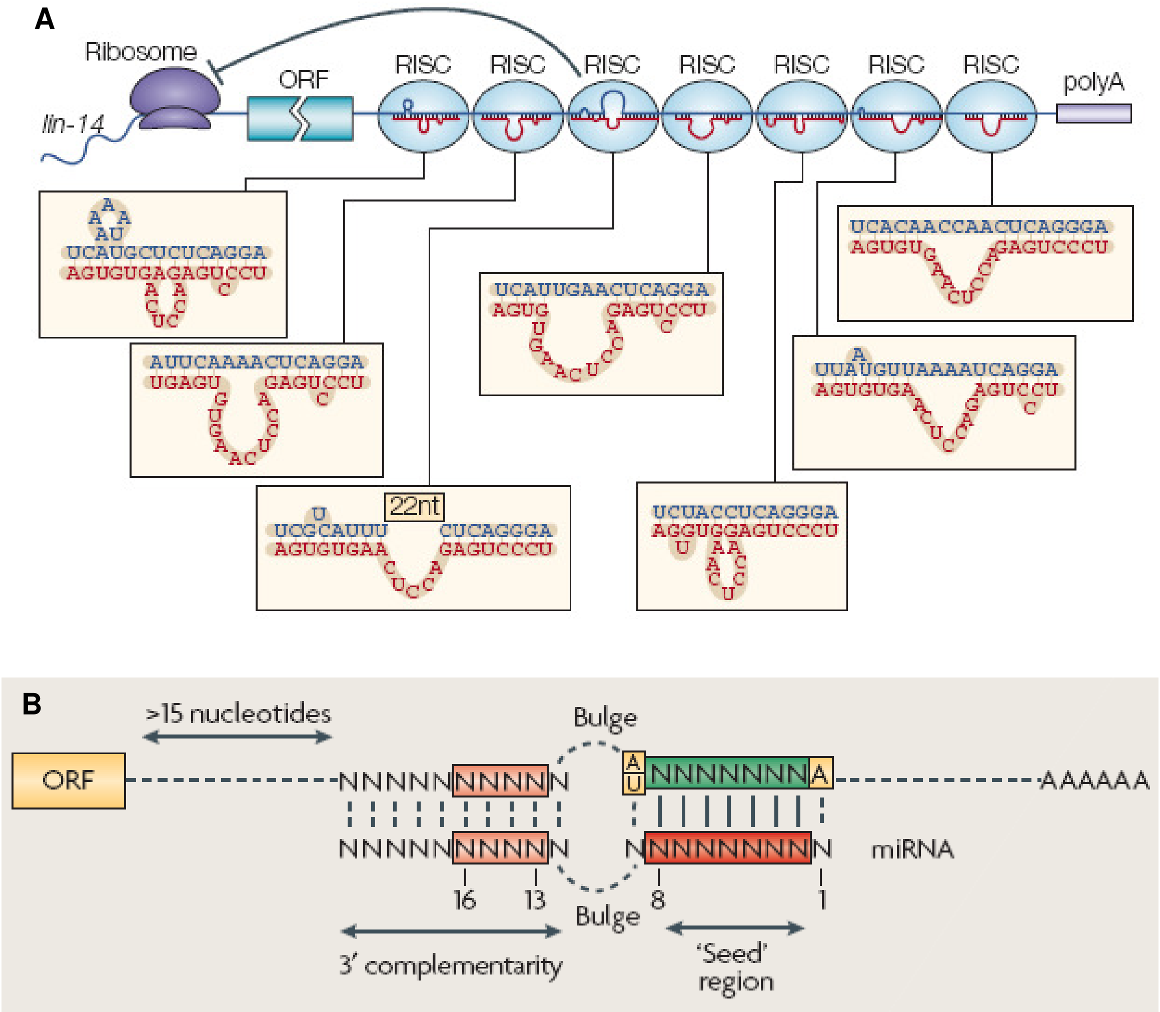


Fig. 1. The interaction between miRNAs and mRNAs.

(A) Predicted *lin-4-lin-14* RNA duplexes in the 3'UTR of the *lin-14* mRNA. *lin-4* (red) is partially complementary to seven sites in the *lin-14* UTR (blue). (B) Principles of miRNA-mRNA interactions. In metazoans, miRNAs base pair imperfectly to the 3'UTR of their target mRNAs. One widely accepted rule for miRNA-target base pairing is perfect and contiguous base pairing of miRNA nucleotides 2 to 7, or 2 to 8 (the 'seed' region, shown in red) to its target (green). Bulges or mismatches are typically present in the central region of the miRNA-mRNA duplex. (adapted from Wightman et al. 1993, He and Hannon. 2004, and Filipowicz et al. 2008).

miRNAs can also disrupt specific developmental processes. For example, *miR-196a* has been reported to negatively regulate the expression of homeobox (Hox) RNA, *Hoxb8*, for embryonic patterning of the posterior trunk and the tail of mice[18].

1.3.2 miRNAs and cancer

In recent years, miRNAs in many ways have also been linked to cancer. One of the earliest evidence showed that *C. elegans let-60/RAS* is a target of the *let-7* miRNA family. It was observed that upon overexpression of *let-7* family members, the multivulva phenotype of *let-60/RAS* mutants can be suppressed[19]. *RAS*, an oncogene that is activated in many human cancers, contains multiple *let-7* binding sites in its 3'UTR. In many cases of lung cancer, *let-7* is significantly reduced, while RAS proteins are highly expressed. Studies in human lung cancer cell lines and xenografts showed that upon expression, *let-7* can act as a tumor suppressor and inhibit the growth of these cancerous cells by inducing cell cycle arrest and cell death[19-22].

Other examples of miRNAs implicated in cancer are *miR-15a* and *miR-16a* in chronic lymphocytic leukemia (CLL). In CLL, the oncogene anti-apoptotic B cell lymphoma 2 (BCL2) is overexpressed while *miR-15a* and *miR-16a* are either deleted or downregulated. *In vivo* and *in vitro* studies have reported that *miR-15a* and *miR-16a* are tumor suppressors and can interact with Bcl2 mRNA to suppress its expression and induce apoptosis[23, 24]. Such findings indicate that loss of the miRNAs in CLL cases results in the overexpression of BCL2, ensuring cell survival by inhibiting cell death[24].

1.3.3 miRNAs and maternal-to-zygotic transition

While sometimes subtle in their functions, other miRNA-mediated events can have a more drastic effect on gene networks. In zebrafish, *miR-430* is a key regulator of maternal-to-zygotic transition (MZT), the period in which embryos no longer rely on maternally provided transcripts and activate zygotic gene expression[25]. *miR-430* is a zygotic miRNA expressed during early zebrafish development. At the onset of zygotic transcription, *miR-430* represses the expression of hundreds of maternal mRNAs, targeting them for deadenylation and destabilization[26]. In the absence of *miR-430*, target mRNAs accumulate in the embryo and are not cleared efficiently, resulting in morphogenic defects that include severe defects during gastrulation, brain formation and heart development[26]. These findings not only demonstrate that *miR-430* is essential for proper morphology, but also provide an insight into the biochemistry underlying miRNA-mediated silencing.

MZT is a conserved process in development and a similar temporal regulation by miRNAs exists in *D. melanogaster*. *miR-309*, an early zygotically expressed miRNA, is also thought to promote the turnover of many maternally deposited mRNAs[27].

1.4 miRNA biogenesis

miRNAs are derived from a primary transcript (pri-miRNA) through sequential processing by two nucleases of the RNase III endoribonuclease family (Fig. 2). These small RNA molecules can originate from a single locus in one transcript, or as a cluster of loci, in which one pri-miRNA with an operon-like organization gives rise to multiple miRNAs[13]. One example of a miRNA cluster is the *miR-35-42* family (Fig. 3). This family consists of the *miR-35-41* cluster, which originates from one locus, and *miR-42* which originates from another locus. All eight miRNA members contain a conserved sequence at positions 2 to 8 from the 5' end that is important for miRNA-target base-pairing and are therefore classified into the same family[28] (see section 1.5). Pri-miRNAs are transcribed from miRNA genes or intronic regions of protein-coding genes in the nucleus by RNA polymerase II and subsequently fold into hairpin structures[29]. The Microprocessor complex, Drosha and its cofactor Pasha/DGCR8, cleaves the hairpin stem loop from the transcript to give rise to a ~70-nt precursor miRNA (pre-miRNA)[30]. The pre-miRNA is then exported out into the cytoplasm by Exportin 5[31]. Once in the cytoplasm, the pre-miRNA stem loop is recognized by another member of the RNase III family, Dicer, which cleaves the hairpin loop to create an RNA duplex with 5'-monophosphate ends and 2-nt 3' overhangs[16]. In *C. elegans*, the miRNA-specific Argonautes (Agos) are required not only for the effector step of silencing, but for the

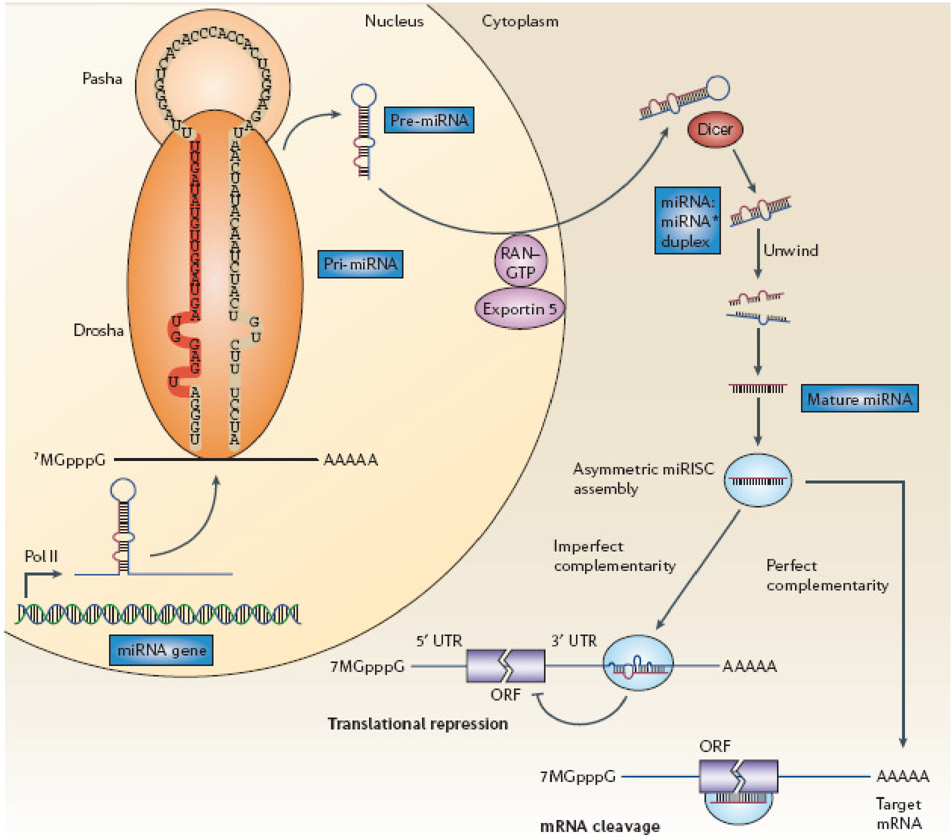


Fig. 2. The biogenesis of miRNAs

miRNA genes are generally transcribed by RNA polymerase II in the nucleus to form large pri-miRNA transcripts, which are m^{7}GTP-capped and polyadenylated[32, 33]. Pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the ~70-nt pre-miRNA precursor product, which is then exported into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient ~22-nucleotide RNA duplex. This duplex is then loaded into miRISC, which includes the Ago proteins, and only one of the single-stranded 22-mer is preferentially retained in this complex. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways depending on the degree of complementarity between the miRNA and its target. In plants, miRNAs bind to their mRNA targets with perfect complementarity, inducing target-mRNA cleavage (lower right). In metazoans, miRNAs typically bind to mRNA targets with imperfect complementarity to block target gene expression post-transcriptionally (lower left) (adapted from Esquela-Kerscher and Slack. 2006)[34].

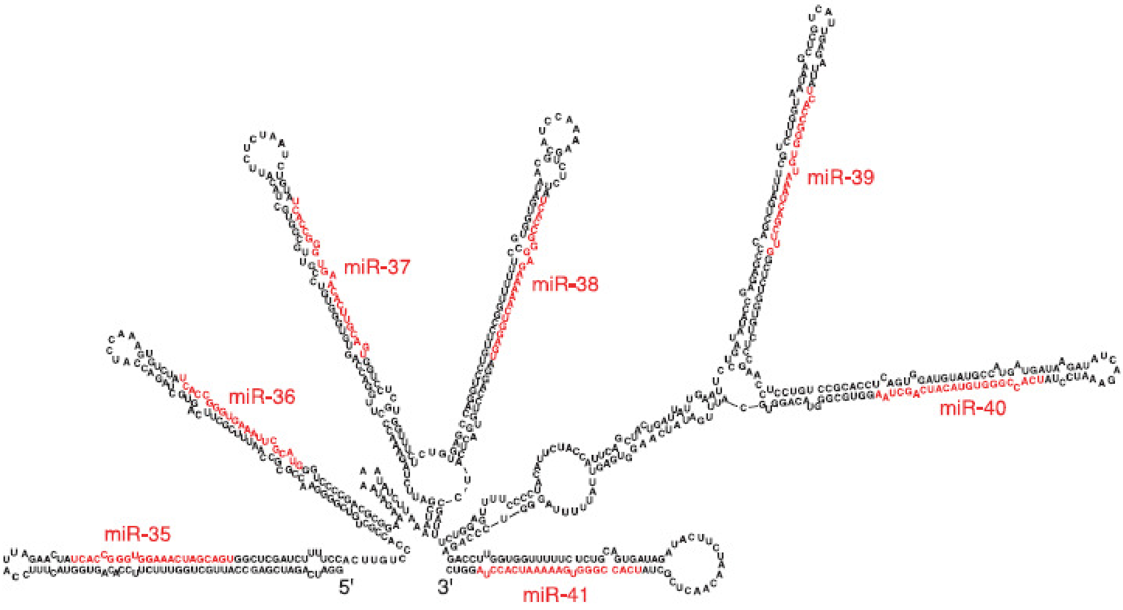


Fig. 3. The *miR-35-41* cluster.

The *miR-35-41* cluster consists of seven miRNAs derived from a single operon (or cluster) on chromosome II. All seven members share the same seed region at positions 2 to 8 from the 5' end of the miRNA. Of the seven miRNA members, *miR-35* is the most abundant. Red sequences denote the mature miRNA sequence (adapted from Lau et al. 2001).

maturation of miRNAs as well. The *A*rgonaute-*l*ike *g*enes 1 and 2 (ALG-1 and ALG-2, ALG-1/2) proteins work in conjunction with Dicer to form 20-25 nt miRNAs. In the absence of ALG-1 and ALG-2, the pre-miRNA accumulates and fails to accumulate as single-stranded short 20-25 nt RNAs[35]. Following miRNA processing, one strand of the duplex is then selected to become the mature miRNA and is loaded onto a *miR*NA-*i*nduced *s*ilencing *c*omplex (miRISC) to form a miRNA-ribonucleoprotein (miRNP) complex. Agos make up the core of the RISC and the number of Agos encoded by the genome and their roles in RNAi vary between species. *C. elegans* express 27 Agos, of which only ALG-1 and ALG-2 are known to function in the miRNA pathway[36]. In addition to Agos, miRNPs contain other proteins that assist the Argonautes in translational repression and/or mRNA destabilization.

1.5 miRNA-mRNA interaction

The specificity of miRNA action on their targets is achieved by sequence-specific interactions between the regulatory miRNA and its target mRNA. In plants, miRNAs bind to their targets with perfect complementarity, inducing rapid mRNA cleavage through the ribonuclease activity (termed “Slicer”) of Agos[37]. On the other hand, in metazoans the majority of miRNAs bind only partially to their targets. Binding of a miRNA with its target mRNA occurs mainly through a region located at the 5’ end of miRNAs, a portion known as the “seed” region. The seed region is the main determinant for target recognition and consists of the nucleotides at positions 2 to 7 or 2 to 8 of the 5’ end of miRNAs[38, 39]. The seed binds perfectly to its complementary site on the 3’UTR of its mRNA target via Watson-Crick base-pairing (Fig. 1B). Multiple miRNA sites for a specific miRNA can be present on any given mRNA’s 3’UTR, while in some cases, one miRNA can merely fine-tune the activity of one target mRNA. A computational approach to study the interaction between multiple miRNA binding sites for a single miRNA species showed that translational repression increases proportionally with the number of miRNA binding sites[39]. Although a given mRNA can be subjected to regulation by more than one miRNA, the effect of different miRNAs binding a single target are not well known, adding more complexity to this gene regulatory network.

1.6 Models for miRNA-mediated gene repression

Despite the broad interest in studying miRNA mode of action, the exact mechanism(s) by which miRNAs inhibit protein synthesis remains controversial. MiRNAs have been shown to modulate mRNA translation and stability via the following mechanisms (summarized by Fig. 4): (i) repression at the level of translation; (ii) repression at post-initiation steps; (iii) premature ribosome dissociation; (iv) mRNA degradation; and (v) poly(A) removal[40-42]. The first mechanistic details were observed in *C. elegans* by Olsen and Ambros, using the *lin-4* miRNA and its target *lin-14* mRNA as a model. Olsen and Ambros noticed that the repressed *lin-14* mRNA remained associated with polysomes while no changes in mRNA levels were observed, suggesting that miRNAs inhibit translation at steps after the initiation phase[6]. Since then, Olsen and Ambros' model has been challenged by a growing number of groups. For example, studies have also shown that miRNAs inhibit translation at the initiation step. One study based on bioinformatic and biochemical approaches revealed that the mammalian Ago involved in miRISC, Ago2, contains a motif similar to the m^7GTP cap-binding domain of the cap-binding protein, eIF4E. The authors proposed that the cap binding-like motif could interact with the m^7GTP cap on mRNAs, inhibiting eIF4E from recognizing the cap and preventing the recruitment of the translation initiation complex[43]. Although this model has been challenged since by independent studies, others have reported findings that also support miRNAs targeting the initiation step of translation. For example, upon addition of the cap-binding complex eIF4F to an *in vitro* system derived from mouse Krebs-2 ascites that recapitulates miRNA-mediated silencing, translation of a reporter mRNA containing *let-7* binding sites was stimulated, supporting the model that the miRNA machinery interferes with translation initiation, specifically by targeting the m^7GTP-cap recognition process[44].

Although Olsen and Ambros did not notice any changes in *lin-14* mRNA level upon *lin-4* targeting, numerous studies over the years have shown the opposite, suggesting a mechanism for miRNA-mediated silencing involving a mild-to-moderate mRNA destabilization. With the same miRNA-mRNA model, *lin-4* miRNA and *lin-14* mRNA, Bagga et al. were among the first to suggest that miRNAs can promote mRNA

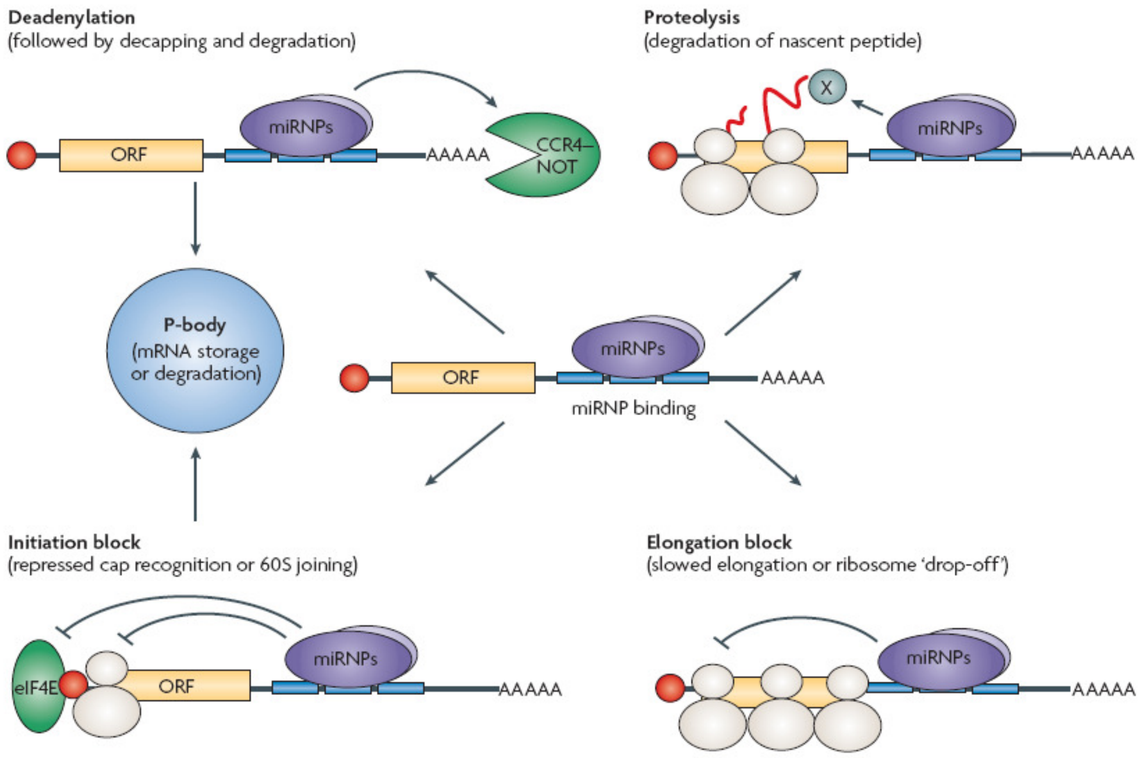


Fig. 4. Possible mechanisms of miRNA-mediated repression in animal cells.

Binding of miRNA-ribonucleoproteins (miRNPs) to mRNA 3′ UTRs is thought to induce deadenylation and decay of target mRNAs (upper left)[26, 45, 46]. Alternatively, miRNPs can repress translation initiation at either the cap-recognition stage or the 60S subunit joining stage (bottom left)[44, 47-50]. mRNAs repressed by deadenylation or at the translation-initiation stage are moved to processing bodies (P-bodies), the sites for mRNA deadenylation, degradation, and temporarily silenced mRNAs[45, 51-54]. The repression can also occur at post-initiation phases of translation, owing to either slowed elongation or ribosome 'drop-off' (bottom right), or proteolytic cleavage of nascent polypeptides (upper right)[55-57]. The m^7GTP cap is represented by a red circle. (adapted from Filipowicz et al. 2008).

destabilization in addition to translational repression, when they observed a significant decrease in *lin-14* mRNA levels *in vivo* by northern analyses upon the expression of *lin-4*[58]. They also showed similar findings with *let-7* miRNA and *lin-41* mRNA target. More recently, deadenylation has also been proposed as a model for miRNA function. *In vitro* studies using miRNA reporters in cell-free extracts often showed poly(A) tail removal of reporter mRNAs associated with translational repression[44, 46, 50]. In line with this evidence, miRISC has been shown to interact with members of the CCF-1/NOT deadenylation machinery[45, 59]. With the increasing wealth of evidence supporting deadenylation as a key mechanism for miRNA action, miRNA-mediated deadenylation is emerging as the favoured model for many.

1.7 mRNA deadenylation and decay

In eukaryotes, deadenylation is often the first and rate-limiting step in mRNA decay. mRNA degradation and deadenylation is thought to occur mostly in processing bodies (P-bodies). P-bodies are mRNP aggregates found in the cytoplasm and are highly enriched in enzymes and proteins involved in mRNA metabolism, as well as translational repressors[60, 61]. Factors that localize to P-bodies include the decapping enzymes DCP1 and DCP2, the 5'→3' exonuclease XRN-1, and the RNA helicase RCK/CGH-1. Recently, several deadenylases, including members of the CCF1/NOT complex, were also found to localize to P-bodies[62]. Another defining component of P-bodies is the GW182 protein. GW182 is a 182 kDa protein that possesses multiple glycine-tryptophan (GW) repeats and an RNA-recognition motif (RRM), which are highly conserved. In mammalians, GW182 has three paralogs, TNRC6A, TNRC6B, and TNRC6C. In *D. melanogaster*, the ortholog is referred to as *Gawky*, or simply as GW182. In *C. elegans*, GW182 proteins lack a recognizable RRM and are referred to as the *A*lg-*IN*teracting proteins (AIN-1 and AIN-2)[63, 64]. AIN-1 and AIN-2 (AIN-1/2) mutants exhibit developmental defects that are similar to animals deficient in ALG-1 and ALG-2, suggesting the *C. elegans* GW182 proteins also participate in the miRNA pathway. *In vivo* studies demonstrated that AIN-1/2 are required for translational repression and mRNA degradation by miRNAs[65, 66]. Immunostaining showed that GW182 proteins colocalize in P-bodies with the decapping enzymes, decapping enzyme activators, deadenylase factors, as well as members of the miRNA machinery[45, 52, 63, 67, 68]. The implications of these findings are that miRNA-mediated translational repression, deadenylation and destabilization may in fact be coupled events. However, direct evidence is still lacking and this remains an area of active research.

1.8 Objectives

The examples listed above present only a few implications of miRNAs in animals. From acting as genetic switches to promoting tumorigenesis, miRNAs are involved in diverse cellular processes. While our knowledge of miRNAs has expanded since their discovery almost a decade ago, the details of the mechanism underlying miRNA action remain unclear.

The purpose of this thesis is to better understand the mechanism for miRNA action and the implications of miRNA-mediated silencing in early animal development. We developed a cell-free extract derived from *C. elegans* embryos to address the following questions: how do miRNAs mediate gene silencing? Do miRNAs employ one mechanism to silence their targets or do different miRNAs use multiple mechanisms depending on the target and the cellular context? What is the impact of maternal and zygotic miRNAs on mRNA stability and poly(A) tail? And can multiple RISCs collaborate on miRNA-binding sites on 3'UTRs to exert silencing effects?

CHAPTER 2: MATERIALS AND METHODS

2.1 *C. elegans* strains and RNAi

The Bristol strain N2 was used as the standard wild-type strain. *C. elegans* were cultured using standard techniques as described[69].

alg-2(ok304) animals were exposed to *alg-1 RNAi*, starting with L2 larvae. RNAi was carried out as previously reported[70, 71].

2.2 Construction of plasmids

RL constructs containing miR-35 sites The open reading frame for *Renilla luciferase* (RL) was cloned in NheI-XbaI sites of pCI neo vector (Promega). A poly(A) tail of 87 nucleotides was cloned into NotI/MfeI. To generate RL reporters containing *miR-35* sites, annealed primers were inserted into XbaI-NotI sites of pCI neo RL to first obtain constructs having one bulged miR-35 site in the 3' UTR. The new insert contains an EcoRI site, which was then digested to insert a new set of annealed primers having three *miR-35* binding sites. A final EcoRI digestion was conducted and a third set of annealed primers was inserted to generate a construct with six complementary *miR-35* sites.

The following pairs of primers were annealed and cloned into pCI neo RL to generate the RL miR-35 reporters with one to six copies of *miR-35* binding sites:

1xmiR-35 fwd: 5'-CTAGAGATTTTTCCCACTGCTAGCCACCCGGTGAATTCGC-3'

1xmiR-35 rev: 5'-GGCCGCGAATTCACCGGGTGGCTAGCAGTGGGAAAAATCT-5'

2xmiR-35 fwd: 5'-AATTGACTGCTAGCCACCCGGTGATTAATACTGCTAGCCACCCGGTGATTAAT-3'

2xmiR-35 rev: 5'-AATTCATTAATCACCGGGTGGCTAGCAGTATTAATCACCGGGTGGCTAGCAGTC-3'

3xmiR-35 fwd: 5'-AATTGACTGCTAGCCACCCGGTGATTAATACTGCTAGCCACCCGGTGATTAATACTGCTAGCCACCCGGTGATTAATG-3'

3xmiR-35 rev: 5'-AATTCATTAATCACCGGGTGGCTAGCAGTATTAATCACCGGGTGGCTAGCAGTATTAATCACCGGGTGGCTAGCAGTC-3' *miR-35 targets 3’UTR cloning* 3’UTR sequences were amplified by PCR from genomic DNA isolated from *C. elegans* embryos. The amplified DNA was then cloned into XbaI/NotI in pCI neo RL. The following primer pairs were used to amplify the UTRs:

C34H3.1 fwd	5’-ATAAACTAGTGCAATGCTTGATTCTACCACA-3’
C34H3.1 rev	5’-TATTGCGGCCGCTAATGGAATCTGTGAGCAACG-3’
hlh-11 fwd	5’-ACTAGTGCCTGACTTTTGACAAATGTAG-3’
hlh-11 rev	5’-GCGGCCGCATTGGTACTCTTGTCTCAGTGG-3’
nhl-2 fwd	5’-ATAAACTAGTGGAGGTTACCCCAATTCCTAT-3’
nhl-2 rev	5’-TATTGCGGCCGCGGGCGAGCTGAAATTCAAATT-3’
R05H11.2 fwd	5’-ATAAACTAGTATTGAATACTTATAGACCTCAAG-3’
R05H11.2 rev	5’-TATTGCGGCCGCTCTAACCGTCTGAATATTATCTG-3’
spn-4 fwd	5’-ATAAACTAGTTCAGTTCAACTGATACGCCC-3’
spn-4 rev	5’-TATTGCGGCCGCTATGGCGAAGCACTTCATTTG-3’
toh-1 fwd	5’-ACTAGTATTCATTTTCTAGTTCTTCTACTC-3’
toh-1 rev	5’-GCGGCCGCAAGACTCAAATGTTTCATTGGG-3’
Y71F9B.8 fwd	5’-ATAAACTAGTATTTTCAGGCTTTCAAGCCCA-3’
Y71F9B.8 rev	5’-TATTGCGGCCGCTTTATAGTTAATAAATTTATTTGATTTA-3’

The sequences of 6xmiR-35 mutant, 6xmiR-52, 6xmiR-52 mutant, and egl-1 3’UTR were cloned as oligonucleotides into pIDTSMART-KAN (IDT):

6xmiR-35 mutant: 5’-TCTAGAACTGCTAGCCACCCAACAAATTGACTGCTAGCCACCCAACAATTAATACTGCTAGCCACCCAACAATTAATACTGCTAGCCACCCAACAATTAATGAATTGACTGCTAGCCACCCAACAATTAATACTGCTAGCCACCCAACAATTAATGAATTCGCGGCCGC-3’;

6xmiR-52: 5’-TCTAGAACTGCTAGCCACCCGGTGAATTGACTGCTAGCCACCCGGTGATTAATACTGCTAGCCACCCGGTGATTAATACTGCTAGCCACCCGGTGATTAATGAATTGACTGCTAGCCACCCGGTGATTAATACTGCTAGCCACCCGGTGATTAATGAATTCGCGGCCGC-3’;

6xmiR-52 mutant: 5’-TCTAGAAGCACGGAAAATGTACAAACGATTGAGCACGGAAAATGTACAAACGTTAATAGCACGGAAAATGTACAAACGTTAATAGCACGGA

AAATGTACAAACGTTAATGAATTGAGCACGGAAAATGTACAAACGTTAATAG CACGGAAAATGTACAAACGTTAATGAATTCGCGGCCGC-3';

egl-1 3'UTR: 5'-TCTAGAGTGATCAAAATCTCCAACTTTTCTCCAATTTGTACCA TGATTTCTCATAATACCCGGTGTTTTTCTTCATTTGTGATTATTTTTCGATCTC TCCGTCTCCAACTCCCCTCAATATTTGTACCATAGTCCTTTATTGCTCATATTT ATCTAATAATAAATATGGTTTTTTTGCGGCCGC-3'.

XbaI and NotI were used to excise 6xmiR-35 mutant, 6xmiR-52, 6xmiR-52 mutant, and egl-1 3'UTR from pIDTSMART-KAN and were subsequently cloned into pCI neo RL in XbaI-NotI sites.

2.3 miRNA expression profiling by northern analysis

Embryos from adults bearing 1-3 embryos per animal (EE) were harvested, and allowed to further develop for 6 hours at 17°C (ME), and 12 hours (LE). Animals were also harvested as synchronous populations of L1, L4 and adult stages.

100-200 ul pellet of *C. elegans* was collected and treated with 1 ml of TRIZOL (Invitrogen) per 100 ul pellet. The pellet was then homogenized with a stainless steel homogenizer at room temperature (30-40 strokes). RNA was extracted and precipitated with isopropanol. To ensure complete removal of genomic DNA, RNA was treated with DNase Turbo (Ambion) for 20 mins at 37°C, followed by phenol/chloroform treatment and precipitation with 0.1 volume 3M NaOAc and 3 volumes of 100% ethanol. 10 ug of RNA was isolated and resuspended in Gel loading buffer II (Ambion) followed by separation on a 15% polyacrylamide/urea gel. The gel was then transferred to a Hybond XL membrane (GE Healthcare) by semidry electroblotting. Membranes were crosslinked by ultraviolet light. Membranes were pre-hybridized for 1 hr at 65°C, and hybridized overnight at 32°C with the following Starfire probes complementary to *miR-35*, *miR-52*, and *miR-58*:

α-*miR-35*: 5'-ACTGCTAGTTTCCACCCGGTGA/3StarFire/-3'
α-*miR-52*: 5'-AGCACGGAAACATATGTACGGGTG/3StarFire/-3'
α-*miR-58*: 5'-ATTGCCGTACTGAACGATCTCA/3StarFire/-3'.

Starfire probes (IDT) were prepared by incubating 1 ul of 0.5 uM Starfire probe with 1 ul template oligonucleotide and 1 ul 10X Starfire reaction buffer for 1 min at 95°C. Reactions were cooled to room temperature for 5 mins. Starfire probes were radiolabeled with [α-^{32}P]-dATP; 6000 Ci/mmol, 20 mCi/ml; Perkin Elmer) and purified by Sephadex G-25 Oligonucleotide Spin Columns (Roche Applied Science).

After hybridization, membranes were washed twice 30 mins with 0.5% SDS and once 15 mins with 1X SSC and 0.2% SDS. Radioactive signals were detected by exposure to BAS-MS 2025 (Fujifilm) and analyzed using a Typhoon Phosphorimager (GE Healthcare). For ribosomal RNA loading control, the gel was stained with ethidium bromide for 15 mins and exposed to ultraviolet light.

2.4 Quantitative reverse-transcriptase PCR of *miR-35*

For analysis of *miR-35* levels throughout *C. elegans* development, 500 ng of purified total RNA from various developmental staged animals (see section 2.3) was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) using the following *miR-35* specific primer (0.1 uM final): 5'-CATGATCAGCTGGGCCAAGAACTGCTA GTT-3'. *miR-35* levels were assessed by real-time PCR on a Mastercycler ep *realplex* (Eppendorf) using SYBR green (Quanta Biosciences). The following primers were used to measure *miR-35* levels: 5'-CATGATCAGCTGGGCCAAGA-3' (*miR-35* universal primer) and 5'-T+CACCGGGTGGAAAC-3' (*miR-35* LNA).

2.5 2'-*O*-Methyl (2'-*O*-Me) pulldown

C. elegans embryonic lysates were first prepared by homogenizing the embryo pellet in ice-cold lysis buffer buffer (25 mM Hepes-KOH pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.5% Triton X-100, 2% RNasin and protease inhibitors) using a stainless-steel dounce homogenizer. The homogenized extract was clarified by centrifugation twice at 13,200 rpm for 10 mins at 4°C. The lysate was then pre-cleared with 20 ul streptavidin beads (equilibrated with one volume of lysis buffer) and 10 ul of an unrelated 2'-*O*-Me oligonucleotide that was not biotinylated (1 uM *miR-1*, Dharmacon). Extract was adjusted to 250 ul with lysis buffer and incubated for 1 hr at

4°C with agitation. Beads were removed with a magnetic rack and the extract was transferred to a clean microtube. 10 ul of biotin-labeled α-*miR-35* 2'-*O*-methyl oligonucleotide 5'-/5Biosg/UUAAUACUGCUAGUUUCCACCCGGUGAUUAAU-3' (1 uM, IDT) was added and the extract was incubated for 1 hr at 25°C with agitation. Following incubation, the extract was centrifuged at 13,200 rpm for 5 minutes, and the supernatant was transferred to a clean microtube. The supernatant was then incubated with 50 ul of Dynabeads M280 (Invitrogen) for 30 mins at 4°C with agitation. The unbound fraction was removed with a magnetic rack, and the beads were washed three times with ice-cold lysis buffer containing 0.1% Triton and 2 mM DTT, followed by three washes without Triton. To detect ALG-1 and ALG-2 associated with the 2'-*O*-Me oligonucleotides, the beads were heated at 65°C for 10 mins in 45 ul SDS loading buffer. 10 ul of the beads were loaded on gel for Western blot analysis. A polyclonal antibody against peptides in the C-terminal region of ALG-1 and ALG-2 was used.

2.6 Preparation of embryonic extracts

Embryos were harvested from large-scale cultures of *C. elegans* nematodes. A typical preparation involved the harvesting of embryos from 30x 50,000 synchronous 150mm plates of animal cultures. Gravid adults were harvested in 1X M9, distributed in 15ml Falcon tubes, and hypochlorited using potassium/sodium freshly prepared hypochlorite solution (0.1 V/V Na hypochlorite from a 5-6% stock, 0.05 V/V from a KOH 5M stock). Hypochlorite treatment was carried out as followed: animal suspensions were treated for 2 mins by mild hand agitation followed by 20 seconds table-top centrifugations at 2,000 rpm, and removal of supernatants. Hypochlorite treatment was carried out until the suspension was completely devoid of adult corpses (3-4 hypochlorite steps necessary). After all used hypochlorite solution was removed, four washes were carried out using M9 saline. On the second M9 wash, M9 was complemented with 100mM HEPES-KOH pH 7.5. Finally, 3 more washes were carried out in double-distilled RNase-free water. After the final centrifugation, additional care was taken to remove all residual liquid. Typically this resulted in 1 to 1.2 ml of stacked embryonic pellets. Pellets were instantly frozen in

15ml Falcon tubes by immersion in liquid nitrogen. Following this step, embryos could be stored at -80°C for at least 1 year (not tested beyond).

Embryonic pellets were rapidly thawed in hand and placed on ice. 0.3 volumes of embryonic pellet of hypotonic buffer [10 mM Hepes-KOH pH 7.4, 15 mM KCl, 1.8 mM $Mg(OAc)_2$, 2 mM DTT] were used to resuspend the pellet. The slurry was then transferred in a clean, pre-chilled Kontes dounce homogenizer. 30-40 strokes of homogenization were carried out on ice, by series of 10 to allow cooling between series of strokes. The breaking of embryos was monitored by visual inspection of 0.5 ul aliquots on a glass slide using a dissection microscope.

Following embryonic break down, the slurry was recovered and transferred to an RNase-free microfuge tube. The extract was then centrifuged at 13,200 rpm for 10 minutes at 4°C. Supernatant was recovered and centrifuged once more in the same conditions. An aliquot (2 ul) of the resulting supernatant was saved aside to assess dilution of the extract from the filtration fractionation step (see below).

A size-exclusion chromatography step was absolutely required to obtain translation activity. For this, two methods were used: 1) centrifugation-based and 2) gravity. While both methods yielded translating extracts, the gravity-based method yielded more consistent extracts that were active. Sephadex G-25 Superfine beads (Amersham Bioscience) were washed three times with isotonic buffer, the same solution used to elute the extract, in a 15ml Falcon. The beads, which make up four times the volume of the resulting extract supernatant, were then stacked into 10ml Column-Prep (BioRad) and then washed three times with isotonic buffer, the same solution used to elute the extract. Following the preparation of the column, the supernatant was loaded onto the column, followed by pushing the supernatant into the matrix with 1:1 volume of isotonic buffer (30 mM Hepes-KOH pH 7.4, 100 mM KOAc, 1.8 mM $Mg(OAc)_2$, 2 mM DTT). Multiple elutions (6-7 elutions) were obtained and the protein concentration for each fraction was quantified by Bradford. Average concentration of active fractions ranged from 5 ug/ul to

20 ug/ul. 25-ul aliquots of each fraction were made and flash-frozen, and stored at -80ºC for later use.

2.7 Transcription of reporters

Plasmids were transcribed *in vitro* using Megascript® T7 Transcription Kit (Ambion) to produce transcripts capped with the GTP-analog (40 mM), m^7(3'-*O*-methyl)G(5')ppp(5')G anti-reverse cap analog (ARCA) (Ambion). ApppG-capped mRNAs were synthesized using ApppG (New England Biolabs) instead of ARCA. Following transcription, template DNA was digested by incubating it with DNaseI for 30 mins at 37ºC. The resulting mixture was purified by phenol/chloroform extraction and Sephadex RNA Spin columns (Roche Applied Science). The optical density was obtained at 260 nm and the quality and size of the transcripts were verified using 4% polyacrylamide-urea denaturing gel.

2.8 *In vitro* translation assays

The translation mixture was prepared by pooling the following solutions per 1x reaction of 12.5 ul:

0.5 ul of 2.5 mM Spermidine, 0.75 ul of 1 mM amino acids, 0.3 ul of 1 M HEPES-KOH (pH 7.5), 1.6 ul of 10 mM $Mg(OAc)_2$, 0.156 ul of 2 M KOAc, 0.25 ul of 5 ug/ul calf liver tRNA, 0.03 ul RNasin (24.1 U/ul, Amersham Bioscience), 0.21 ul of 1 M Creatine phosphate, 0.34 ul of 3 ug Creatine phosphokinase, 0.25 ul of a 50x mixture of ATP and GTP (0.8 mM ATP and 0.2 mM GTP stock), and 2.114 ul MilliQ water. KOAc and $Mg(OAc)_2$ concentrations need to be adjusted for optimization experiments. 5 ul of *C.elegans* embryonic extract was then added. For each reaction, the translation mixture was aliquoted into individual microfuge tubes and 1 ul of RNA (10 ng) was added, volume and concentration of RNA varied with the type of experiment. Reactions were incubated at 17ºC for 0 to 3 hours. The levels of luciferase at various time points were determined using the Dual-Luciferase® Reporter Assay System (Promega). 2 ul of translation reaction was added to 25 ul Firefly luciferase (FL) buffer and measured with the luminometer (Lumat LB) following a 10 s reading. 25 ul of Renilla luciferase (RL) substrate 1x was then added to quench FL activity and to assay for RL instead. The RL proteins synthesized *in vitro* were analyzed by Western blot using a monoclonal antibody against RL (Chemicon International).

To assay for miRNA activity, *C. elegans* embryonic extract was pre-incubated with 2'-*O*-Methyl (2'-*O*-Me) oligonucleotides (Dharmacon) prior to mRNA addition for 30 minutes at 17°C. The following 2'-*O*-Me miRNA inhibitors were designed as antisense oligonucleotides to the mature miRNAs according to Wormbase registry (www.wormbase.org):

α-*miR-1*:	5'-UCUUCCUCCAUACUUCUUUACAUUCCAACCUU-3'
α-*miR-35*:	5'-UUAAUACUGCUAGUUUCCACCCGGUGAUUAAU-3'
α-*miR-52*:	5'-UUAAUAGCACGGAAACAUAUGUACGGGUGUUAAU-3'
α-*miR-58*:	5'-UUAAUUGCCGUACUGAACGAUCUCAUUAAU-3'

2.9 mRNA stability and deadenylation assays

Radiolabeled RNA ([α-^{32}P]-UTP; 800 Ci/mmol, 20 mCi/ml; Perkin Elmer) was transcribed *in vitro* (Ambion MaxiScript Kit) and 0.1 ng was incubated in *C.elegans* embryos in a total volume of 12.5 ul per reaction in the absence or presence of 50 nM (except where indicated) *miR-35*, *miR-52*, *miR-58*, or *miR-1* 2'-O-Me. 12.5 ul aliquots were withdrawn at specific time points and placed in TRIZOL reagent (Invitrogen). The extracted RNA was loaded on a 4% polyacrylamide/urea gel. The gel was dried, exposed to autoradiography, and analyzed using a Typhoon Phosphorimager (GE Healthcare).

2.10 RT-PCR amplification of *miR-35* targeted reporters

Radiolabeled RNA from *C. elegans* embryonic extract was extracted with TRIZOL (Invitrogen) and precipitated in isopropanol with 1 ul of Glycoblue (Ambion). RNA was resuspended in RNA ligation solution and ligated overnight with a "miRNA universal linker" (New England Biolabs) at 4°C. Ligation products were purified and reverse transcribed with Superscript III (Invitrogen) and amplified using Titanium DNA polymerase (Clontech). PCR products were cloned and sequenced.

CHAPTER 3: RESULTS

3.1 Bulk miRISC programming by the maternal *miR-35-42* and zygotic *miR-51-56* families in *C. elegans* embryos

To study the mechanisms of miRNA-mediated silencing in embryos and their impact on early development, we chose to investigate the function of two abundant classes of miRNAs expressed in *C. elegans* embryos. The *miR-35-42* family consists of 8 miRNAs driven from two loci: *miR-35-41* is expressed as a single operon (or cluster), while *miR-42* is expressed from a separate locus on chromosome (chr.) II (Fig. 3 and Fig. 5A). The *miR-51-56* family is made up of *miR-51* and *miR-53*, which are expressed as an operon (chr. IV), *miR-52* (a separate locus on chr. IV), and *miR-54-56* (derived from another operon on chr. X) (Fig. 5A). Both the *miR-35-42* and the *miR-51-56* families were reported to be highly expressed in the embryo based on cloning and deep sequencing analyses[13, 14, 72, 73]. To refine the expression profile of these miRNAs, we re-visited their profiles using northern blot and qRT-PCR (Fig. 5B and C). As previously reported, total RNA preparations at the non-permissive temperature (25°C) of *glp-4(b2)*, mutants depleted of germline cells, resulted in a complete loss of *miR-35* expression[13]. Curiously, we noticed that pre-*miR-35*, but not the mature form, accumulated in L4 animals, suggesting regulated processing by Dicer (DCR-1 in *C. elegans*). The mature form of the miRNA is present in *fem-1* animals, which are impaired in male germline development, at the non-permissive (25°C) temperature, indicating that processing of pre-*miR-35* occurs during the late stages of germline development, but prior to fertilization (Fig. 5B). Noticeably, the expression of *miR-35* is strongest in the early embryonic preparations, but its expression decreases rapidly and is almost completely lost by the L1 stage. In contrast, *miR-52* expression increases during the maturation of embryos and is strongest in L1 stage preparations, which is consistent with zygotic transcription accounting for most of its expression (Fig. 5C). The expression of another highly abundant miRNA, *miR-58*, was very weak in the early embryonic population, and also appeared to peek at L1 stage (Fig. 5D). These results indicate that the expression of the most abundant miRNA families in the embryo, maternal or zygotic, is strictly regulated at transcriptional and post-

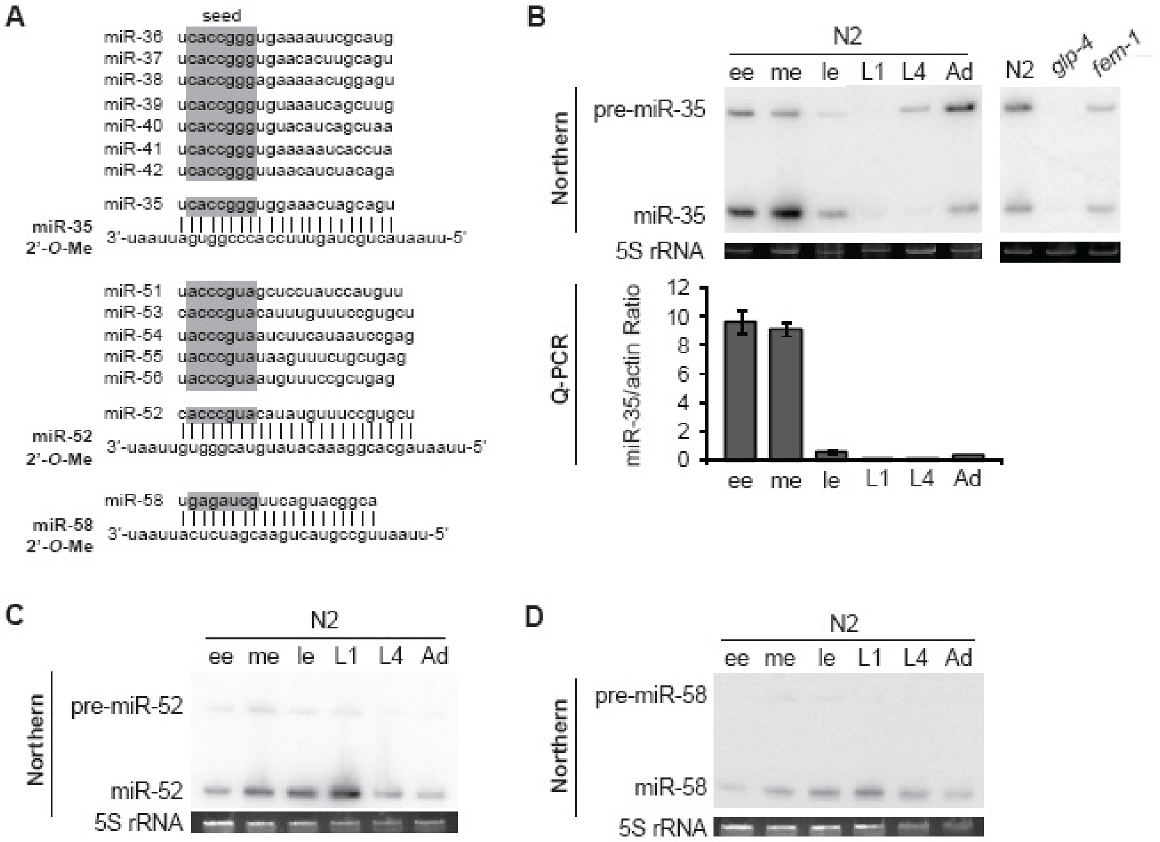


Fig. 5. Expression profile of maternal *miR-35*, and zygotic *miR-52* and *miR-58* RNAs in *C. elegans*.

(A) miRNAs and 2'-*O*-Me oligonucleotides used in this study. The seed region for each miRNA is highlighted in gray. (B) Expression profile of *miR-35* by northern and real-time PCR analysis. Results are presented as the mean from triplicate experiments and error bars indicate standard deviation. (C and D) Expression profile of *miR-52* and *miR-58*, respectively, by northern analysis. Total RNA from developmentally staged wild-type *C. elegans* was isolated from early-stage embryos (ee), middle-stage embryos (me), late-stage embryos (le), L1-, L4-, and adult-stage animals. Ethidium bromide staining of 5S ribosomal RNA (rRNA) served as loading controls.

transcriptional levels.

We next wished to determine the effective concentration of the *miR-35-42*-programmed RISC in embryos. Based on miRNA-specific qRT-PCR, we estimated the concentration of *miR-35* in our mid-embryo preparations to approximately 3 to 8 nM depending on the preparations (data not shown). *miR-35* is the most abundant species of the family based on independent approaches[13, 72, 73]. In comparison, the concentration of *miR-35* is roughly 50 times higher than the concentration of the two most abundant *let-7* miRNAs (*let-7b* and *let-7f*) in Krebs extracts, and approximately 200 times more than *let-7* in HeLa cells[44]. Intrigued by the abundance of these miRNAs, we examined whether they reflect a high abundance and functional miRISC in embryos. For this, we utilized a miRISC affinity pulldown method, based on non-hydrolyzable 2'-*O*-Methylated (2'-*O*-Me) and biotinylated oligonucleotide complementary to *miR-35-42* miRNAs (Fig. 6A)[74]. Pulldown in embryonic lysates was effective against the *miR-35-42* family, as indicated by western blot on the Argonautes ALG-1 and ALG-2 (Fig. 6B). The pool of *miR-35-42* miRNAs was effectively depleted, even for the most diverging family members, although the depletion was less than complete (data not shown). We noticed that ALG-1 migrates as multiple species in western blots, indicating possible splicing isoforms, or post-translational modifications (PTMs). In comparison to *let-7* pulldown, our negative control which did not bring down any significant amount of RISC, a considerable amount of *miR-35-42* RISC was pulled down, suggesting a large fraction of endogenous embryonic ALG-1 and ALG-2 pool is programmed by the *miR-35-42* family (Fig. 6B). ALG-1 and ALG-2 were also pulled down by anti-*miR-52* and *miR-58* baits in middle embryo-staged preparations, although to a lesser extent compared to the *miR-35-42* family (Fig. 6C). We conclude that a few miRNA families account for a large fraction of the programmed RISC in *C. elegans* embryos.

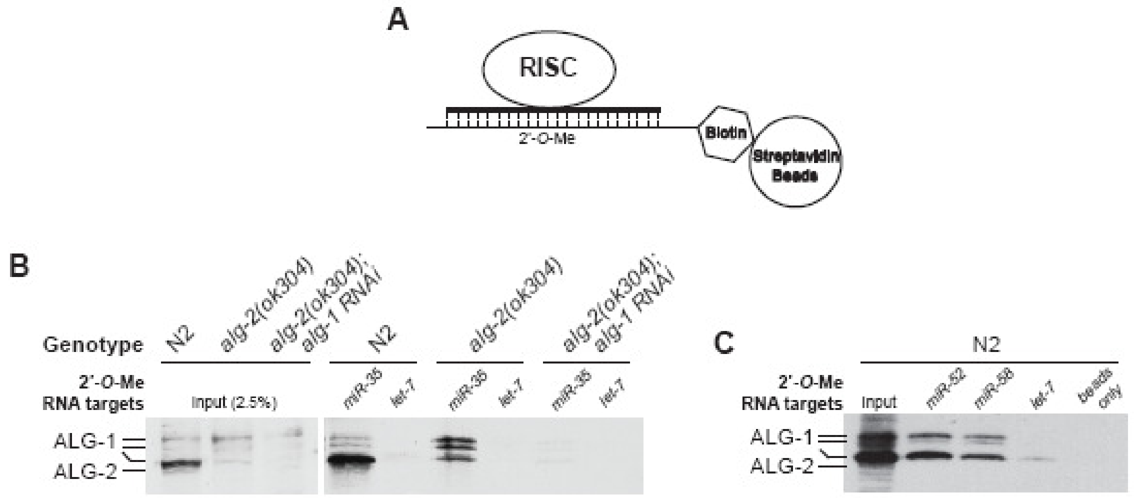


Fig. 6. Characterization of miRISC in *C. elegans* embryos.

(A) Schematic representation of the miRISC 2'-*O*-Me pulldown. (B and C) Western blot of ALG-1 and ALG-2 in extracts and the affinity-purified miRISC. Extracts prepared from wild-type (N2), *alg-2(ok304)*, or *alg-2 (ok304); alg-1 RNAi C. elegans* embryos were incubated with either α-*miR-35*, α-*miR-52*, α-*miR-58* or α-*let-7* 2'-*O*-Me. The associated proteins were probed with a polyclonal antibody against ALG-1 and ALG-2.

3.2 Cap and poly(A) tail synergy in *C. elegans* embryos

To assay for miRNA-mediated silencing by these families of miRNAs, we developed the first cell-free translation system from *C. elegans* embryos capable of initiating translation on exogenous transcripts. Under optimal conditions, the embryonic lysate sustained translation of our mRNA reporters, Firefly and Renilla luciferase (FL and RL, respectively, Fig. 7B), for at least 6 hours (Fig. 7C) with a near-linear capacity of translation on mRNA reporter concentrations reaching up to 26 uM for RL for typical preparations (Fig. 7D). Next, we examined the influence of 5' and 3' terminal structures, specifically the m^7GTP cap and poly(A) tail, on translation efficiency. Translation of mRNAs bearing both a m^7GTP-cap and a poly(A) tail was most efficient, and was greater than the additive contributions of transcripts that bore either a poly(A) tail or m^7GTP-cap (Fig. 7E, m^7GTP-cap/pA+). Hence, our system recapitulates translation and the functional synergy between the 5' cap and 3' poly(A).

3.3 Cell-free silencing by maternal miRNAs in *C. elegans*

To assay for miRNA activity in our system, we examined the translation of RL mRNA fused to a synthetic 3'UTR encoding six copies of a *miR-35-42* binding site (Fig. 8A). Reporters were added to our translation system at a concentration of 1 nM, which is below the concentration of *miR-35* quantified by qRT-PCR, as mentioned previously. Translation of RL 6xmiR-35 was dramatically reduced in comparison to RL mRNA, with activity rapidly slowing down and reaching a plateau at around 1 hr of incubation (Fig. 8C). In contrast, RL mRNA was continuously translated for at least 6 hrs. Addition of a 2'-*O*-Me oligonucleotide antisense to *miR-35* (α-*miR-35*) released the translation of RL 6xmiR-35 (Fig. 8B). De-repression reached 300% when using 50 nM of the *miR-35*-specific inhibitor during a 3-hr translation reaction. We notice that this concentration is consistent with our estimate of the endogenous *miR-35-42* concentration. In contrast, addition of the same concentrations of a 2'-*O*-Me oligonucleotide complementary to the non-related *miR-1* did not affect the translation of RL 6xmiR-35 (Fig. 8B). These results

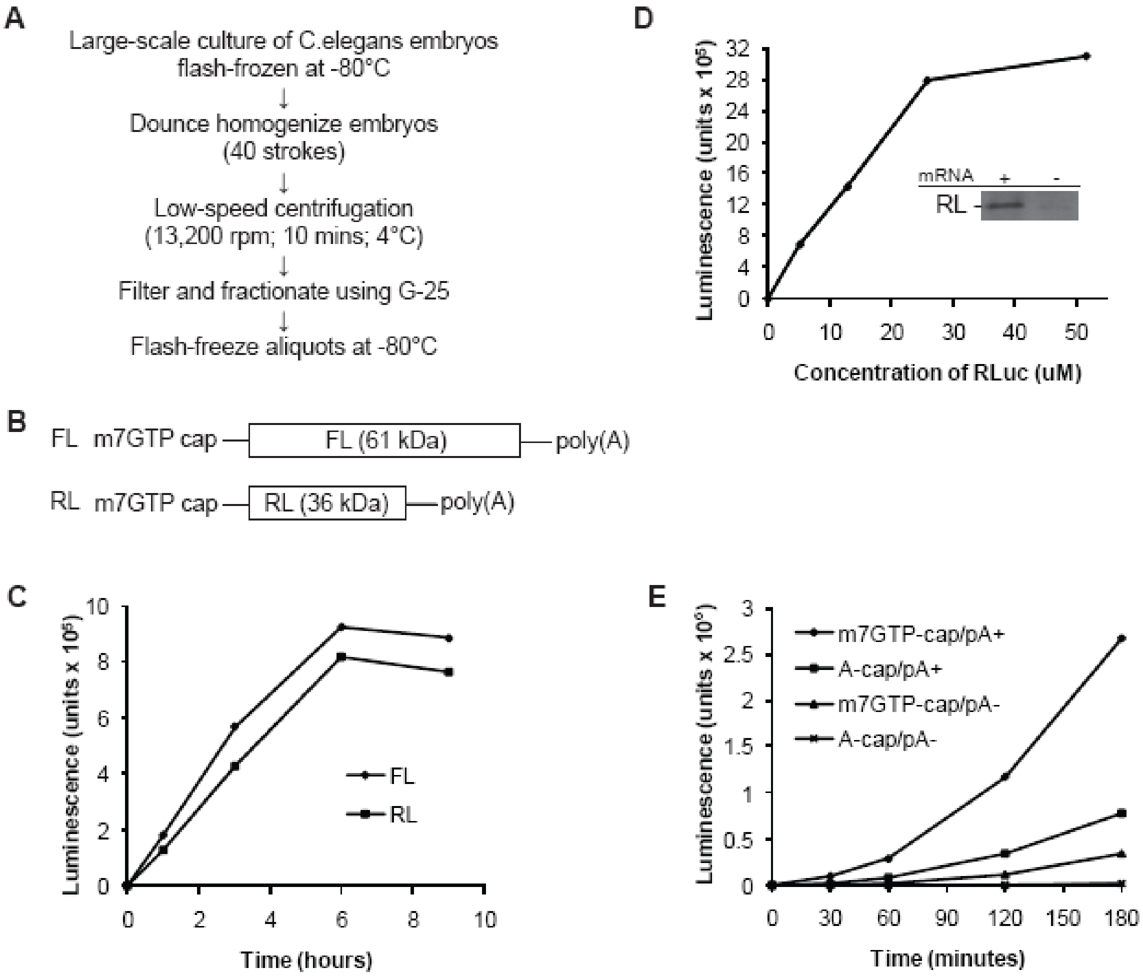


Fig. 7. Cap and poly(A) tail synergy in *C. elegans*.

(A) Outline of the preparation for translation extracts derived from *C. elegans* embryos. (B) Schematic representation of the Firefly luciferase (FL) and Renilla luciferase (RL) reporter mRNAs. (C) Time-course of luciferase translation in *C. elegans* extract. Reporter mRNAs were incubated with supplemented *C. elegans* extract for 0 to 9 hrs. (D) Dose-response curve of the translation activity in *C. elegans* extract as a function of mRNA concentration. Various concentrations of RL reporter mRNAs were incubated with *C. elegans* extract for a period of 3 hrs. (Indent) The RL proteins synthesized *in vitro* were analyzed by western blot using a monoclonal antibody against RL. (E) Cap and poly(A) tail synergy in *C. elegans*. The translation efficiency of RL reporters containing the presence or absence of the physiological 5’ cap and 3’ poly(A) tail was monitored over a 3-hr time course. The results shown are from one set of experiments.

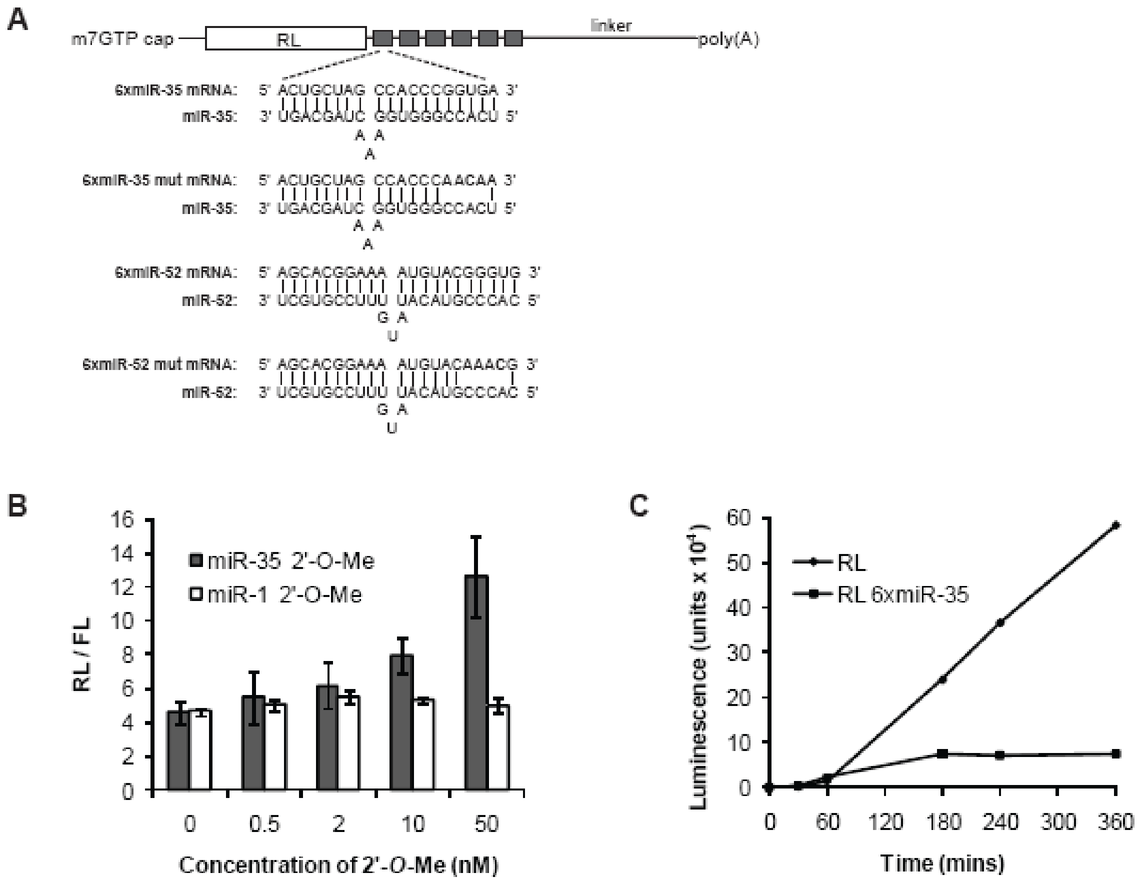


Fig. 8. miRNA-mediated translational repression by maternal miRNAs.

(A) Schematic representation of the RL reporter mRNAs used. Sequences of the *miR-35*- and *miR-52*-binding sites (6xmiR-35 and 6xmiR-52) and mutated binding sites (6xmiR-35 mut and 6xmiR-52 mut, denoted by bold letters) are shown. (B) Translational repression in *C. elegans* extract. Reporter mRNAs were incubated in the presence of 0 to 50 nM 2'-*O*-Me (either α-*miR-35* or α-*miR-1*) for 3 hrs. α-*miR-1* 2'-*O*-Me served as negative controls. Each bar represents the mean from triplicate experiments and error bars indicate standard deviation. (C) Time course of RL and RL 6xmiR-35 mRNAs translation.

show that the *miR-35-42* family potently represses the RL 6xmiR-35 mRNA reporters *in vitro*.

3.4 miRNA-directed deadenylation by maternal and zygotic miRNAs

The course of translation of our reporters shows a rapid and almost complete shut down of translation by the maternal *miR-35-42* family. To determine whether the RL 6xmiR-35 RNA underwent miRNA-directed deadenylation and/or degradation, we examined the integrity of ^{32}P-radiolabeled reporter mRNAs over time after resolution on a denaturing PAGE and autoradiography (Fig. 9). We found RL mRNA reporters to be very stable in our system (Fig. 9A, RL panel). However, when considering the 6xmiR-35 reporter as early as 40 mins of incubation, a shorter RNA species was observed. By 60 mins of incubation, the majority of the reporter had shifted to this species (Fig. 9A, 6xmiR-35 panel, and Fig. 9C N2 panel, denoted by $p(A)_0$). The size of the new RNA species was consistent with deadenylation of our reporter, which was confirmed by cloning and sequencing of the product (see below). Deadenylation was specifically delayed by the addition of anti-*miR-35* 2'-*O*-Me (α-*miR-35* panel), while it was insensitive to the addition of anti-*miR-1* 2'-*O*-Me (Fig. 9A, α-*miR-1* panel). RL 6xmiR-35 mut reporters lacking functional *miR-35* complementary sites remained unaffected by incubation. We wished to further substantiate the specificity of our miRNA-mediated silencing results using extracts genetically depleted of ALG-1 and ALG-2. For this, animal populations were fed on an *E. coli* strain which over-expresses dsRNA against the Argonautes and the resulting (F1) embryos were harvested. These embryos arrested development at, or during enclosure, but translation in this system was at least as efficient, and was most often more potent than wild-type preparations indicating the integrity of the translation machinery in these extracts (data not shown). Western blot and 2'-*O*-Me affinity matrices analyses confirmed an efficient knock down of these proteins in the mutant extract (Fig. 6B *alg-2* and *alg-2; alg-1 RNAi* panels). In these genetically depleted extracts, deadenylation was substantially delayed (Fig. 9C *alg-2* and *alg-2; alg-1 RNAi*, in comparison to N2 controls).

Our observations for miRNA-mediated silencing could be specific for the *miR-35-42*

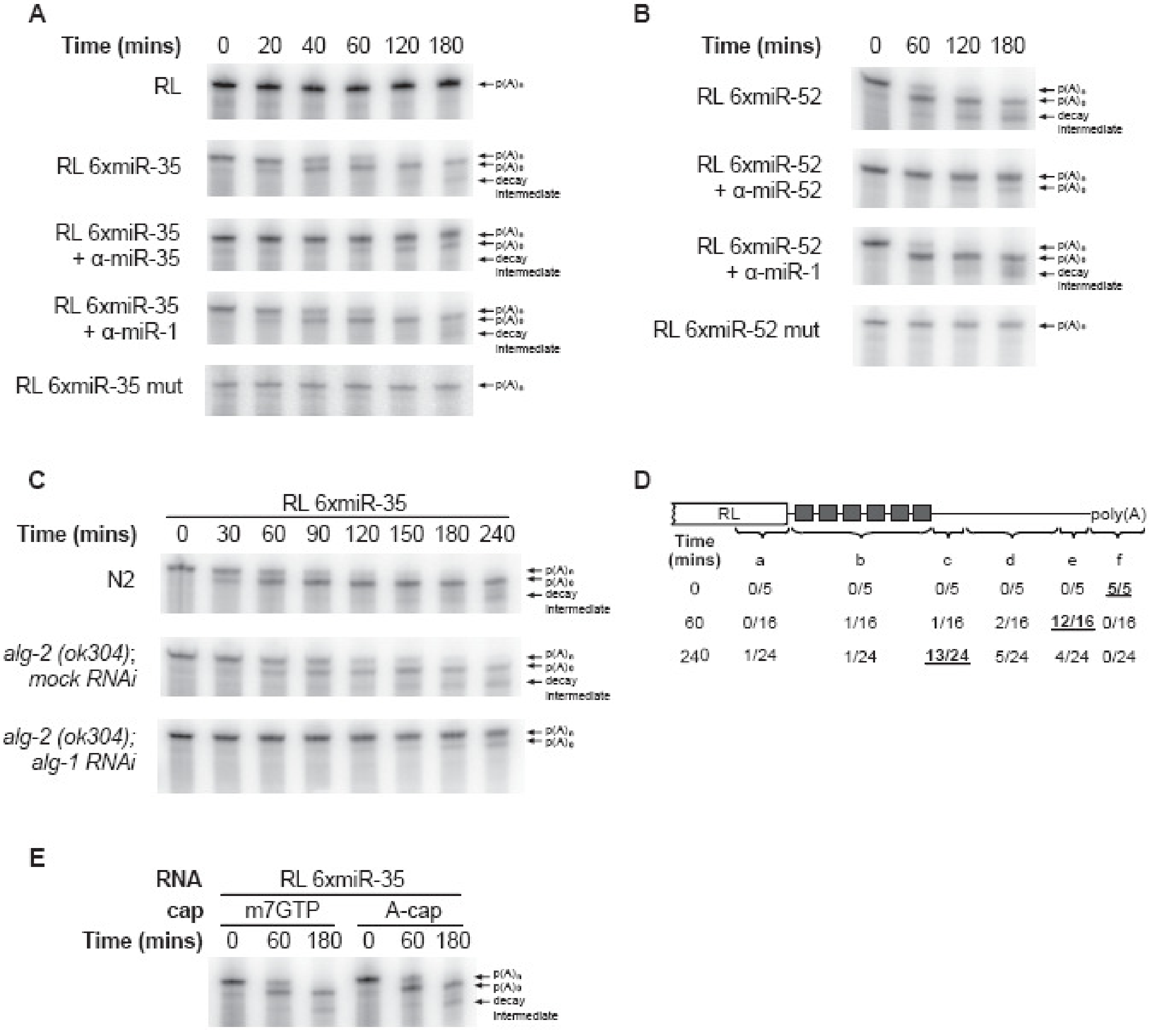


Fig. 9. Maternal *miR-35-42* and zygotic *miR-51-56* direct deadenylation and 3’→5’ decay of reporter mRNAs in *C. elegans* embryos.

(A) Time course of RL, RL 6xmiR-35, and RL 6xmiR-35 mut mRNA deadenylation by the maternal *miR-35-42* family. Reporter mRNAs were incubated in the presence or absence of 50 nM 2’-*O*-Me oligonucleotides (either α-*miR-35* or α-*miR-1*). α-*miR-1* 2’-*O*-Me served as a negative control. (B) Time course of RL 6xmiR-52 and RL 6xmiR-52 mut mRNA deadenylation by the zygotic *miR-52* miRNA. (C) Time course of RL 6xmiR-35 in extracts prepared from wild-type (N2), *alg-2(ok304); gfp (mock) RNAi*, or *alg-2(ok304); alg-1 RNAi C. elegans* embryos. (D) Schematic diagram of the cloning of PCR-amplified RL 6xmiR-35 3’RACE products. Sequences were divided among the following regions: a. within the RL open reading frame, b. between the miRNA binding sites, c. within the first 40 nts downstream of the miRNA binding sites, d. within the middle region of the 3'UTR, e. within less than 25 nts 5' of the poly(A) tail, f. within the poly(A) tail. Bold and underlined numbers indicate the highest fraction of clones at one specific time point. (E) Time course of RL 6xmiR-35 mRNA deadenylation and decay in the presence of m^7GTP cap or A-cap. Deadenylation and stability of the reporter mRNAs were monitored by autoradiography. Full-length and deadenylated mRNAs, and the decay intermediates are marked on the right of each figure.

family, or to maternally contributed miRNAs. We therefore examined the fate of the *miR-51-56* reporter in the same assays. Deadenylation of the *miR-51-56* reporters occurred slightly faster than reporters for the *miR-35-42* family (Fig. 9B, and compared to Fig. 9A at 60 mins), and was almost completely prevented by a sequence-specific 2'-*O*-Me oligonucleotide, or by mutation of the seed-complementary sites (Fig. 9A, C, 6xmiR-35 and 6xmiR-52 mut panels). We conclude that the maternal *miR-35-42* as well as the zygotic *miR-51-56* families direct potent and sequence-specific deadenylation in *C. elegans* embryonic lysates. We notice that the time frame of deadenylation closely parallels the course of translation repression (Fig. 8C). Taken together with the major requirement for a poly(A) tail for translation by this extract, this evidence suggests that deadenylation likely accounts for a major part, if not entirely, in the repression of our reporters.

3.5 Slow, uncoupled miRNA target decay via 5'-cap-independent 3'→5' processing

Deadenylation by miRNAs is thought to result in the rapid destabilization of target transcripts[26, 58]. Initial observation of miRNA-mediated destabilization has been particularly well supported where zygotic miRNAs mediate the rapid turnover of maternal mRNAs in zebrafish and *D. melanogaster*[26, 27]. In our system, however, fully deadenylated reporters proved remarkably stable. Upon close examination of our *miR-35-42* and *miR-51-56* deadenylated reporter autoradiograms, we noticed the appearance of a shorter RNA species around or at 2 hrs of incubation (Fig. 9A, B, and C, see decay intermediate arrows). This intermediate was generated in a miRNA- and/or deadenylation-dependent manner as cognate anti-*miR-35* and anti-*miR-52* 2'-*O*-Me, or genetic depletion of ALG-1/2 prevented the accumulation of this decay intermediate (Fig. 9A, B, and C). Sequencing of multiple independent clones of the recovered reporter mRNA indicated that while the vast majority of reads terminated at, or very near the poly(A) tail site at the 60-min time point (Fig. 9D, regions d and e), reads from clones recovered after 240 minutes clustered closely in the 3' region bordering the miRNA-binding site repeats (Fig. 9D, region c). We noticed that the continuous removal of sequences further upstream to the poly(A) tail over time suggests the involvement of a 3’→5’ exonuclease activity in reporter decay.

While a number of different mechanisms have been proposed wherein deadenylation is coupled to the rapid turnover of miRNA targets, the link between miRNA-directed deadenylation and mRNA decay is still elusive. To clarify this link, we further investigated the decay process in our system. Because ‘miRNA-mediated decay’ is used to describe a number of distinct phenomena in the literature, for our report here we define the term ‘decay’ as the degradation of mRNA sequences upstream to the poly(A) tail. To address whether de-capping is involved in the decay of our reporters, we generated A-capped reporters (not recognizable by canonical de-capping enzymes), and examined their fate by autoradiography. The time-course of deadenylation and decay was exactly the same as for the m^7GTP-capped reporters (Fig. 9E). This result indicates that the reporter decay observed does not require de-capping, and hence likely occurs through a 3’→5’ exonuclease activity.

3.6 Widespread deadenylation of embryonic miRNA targets

Our cell-free system recapitulates many of the features of miRNA-mediated silencing *in vivo* including translational repression, deadenylation, and a modest target decay. It also recapitulates the genetic requirements for the core components of the miRISC machinery. However, and like most of the reports describing the mechanisms of miRNA-mediated silencing, our assays thus far relied on artificial miRNA reporters, which are based on arbitrarily positioned repeats of canonical miRNA binding sites. We therefore asked whether we could faithfully recapitulate miRNA-mediated silencing using endogenous 3’UTR sequences. To obtain a measure of if, and how natural UTRs would undergo miRNA-mediated silencing, we undertook a screen to identify natural 3’UTRs that can promote mRNA deadenylation and decay based on the predicted *miR-35-42* targets. In a pilot screen, 13 UTRs predicted to be *miR-35-42* targets (obtained from TargetScan and miRWIP prediction algorithms) were cloned, and transcribed *in vitro* as fusions with RL mRNA. To improve gel resolution, the UTRs were fused to a truncated version of the RL open reading frame (ORF) that cannot be translated (Fig. 10)[59]. These “short transcripts” were then incubated in embryonic lysates, recovered and resolved on a denaturing PAGE, as presented above. Because these UTRs are predicted to be *miR-35-42* family targets, we conducted the same experiments in the presence of anti-*miR-35* or anti-*miR-1* inhibitors as controls (Fig. 10A). A representative sample of the UTRs screened is presented in Fig. 10.

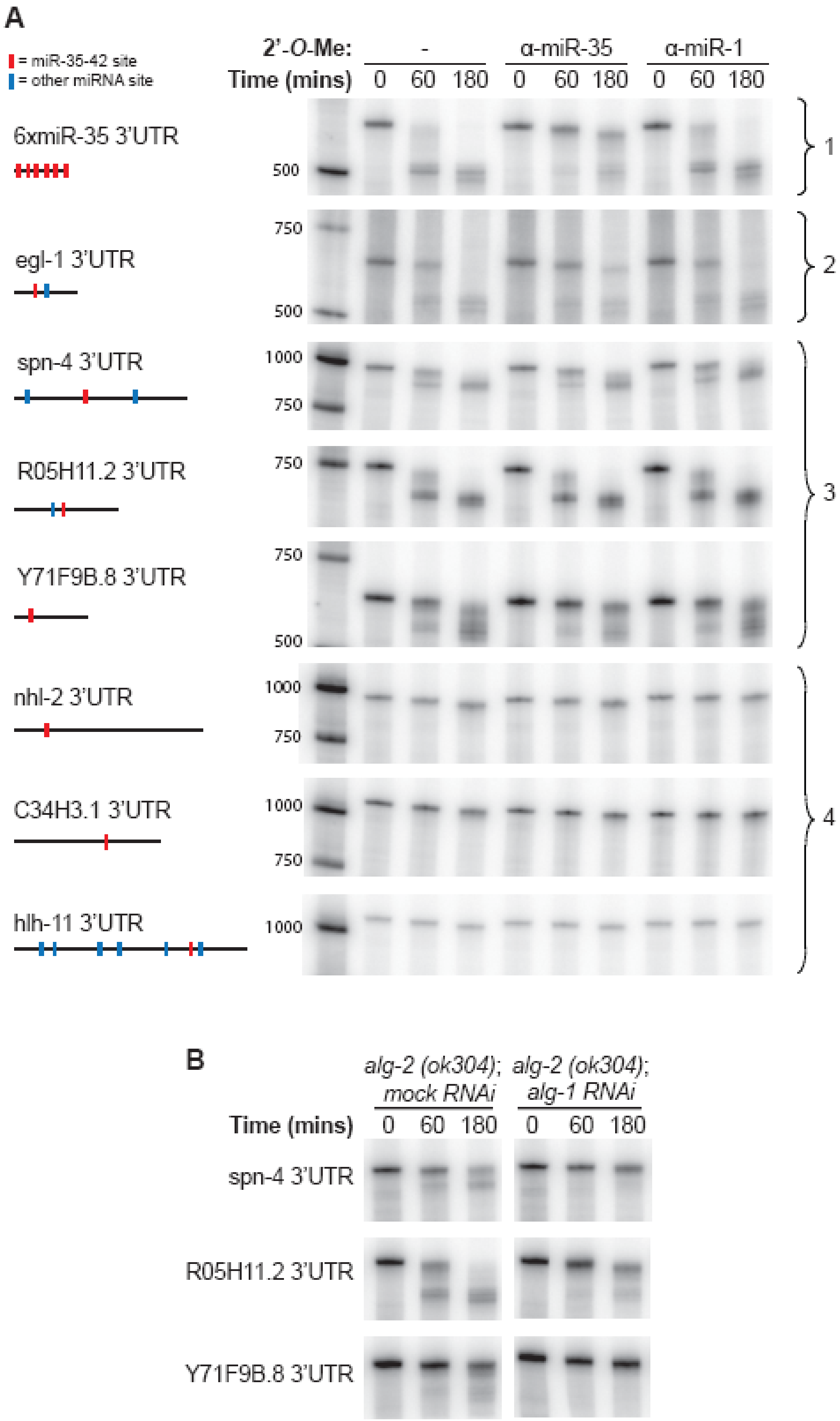
Of the screened UTRs, a total of 6 UTRs did not show signs of robust deadenylation (Fig. 10A, group 4 represents a small subset of the 6 UTRs). Surprisingly, 7 of the 13 UTRs screened thus far were deadenylated (Fig. 10A, groups 2 and 3 shows a small subset of the 7 UTRs). Deadenylation of some of these targets was not substantially prevented by incubation with anti-*miR-35* 2’-O-Me (Fig. 10A, group 3). Since these UTRs were predicted to be targeted by additional embryonic miRNAs (Fig. 10A, blue crossbars), we incubated these target UTRs in the ALG-1/2 depleted extract[73]. In this extract, deadenylation was prevented (Fig. 10B), indicating the involvement of ALG-1 and ALG-2 in the deadenylation of a broad variety of targets in the embryonic context.

Upon addition of the anti-*miR-35* inhibitor, deadenylation of two UTRs, the BH3-only pro-apoptotic *egl-1* and the tolloid/bone morphogenic protein family member *toh-1*, was specifically inhibited (Fig. 10, group 2 and Fig. 11). Since *miR-58* was also predicted to target *egl-1*, we examined whether this zygotically expressed miRNA can also deadenylate *egl-1*. Indeed, deadenylation was strongly impaired upon the addition of anti-*miR-58* 2’-*O*-Me (Fig. 11B). These results identify *toh-1* as a target of the maternal *miR-35-42* family and *egl-1* as a target of both *miR-35-42* and *miR-58*.

3.7 Natural UTR-targeted deadenylation requires miRISC collaboration

We extended the analysis of these UTRs to understand *miR-35-42*’s mechanism of action. *egl-1* and *toh-1* UTRs encode only two sites for miRNAs that are sensibly expressed in the embryo based on the expression data by Stoeckius et al. (Fig. 11A and B, schematic diagram)[73]. Yet, we were able to prevent their deadenylation by inhibiting a single one of these miRNAs at a time (Fig. 11B, only *egl-1* was tested). This suggests that neither of the two separate miRISC-binding sites is sufficient on its own to direct deadenylation. Deadenylation assay of full-length translation RL *toh-1* UTR also showed a block in deadenylation when incubated with cognate anti-*miR-35*, but remained unaffected when incubated with non-cognate anti-*miR-1* at the same concentrations. Together, these results suggest that cooperation between at least two separate miRISC-binding sites is required to drive deadenylation.

To better define this cooperation, we engineered reporter mRNAs bearing 1 to 4 *miR-35-42* binding sites, and examined their fate in deadenylation assays (Fig. 11C). Only when the reporters encode three or more sites was deadenylation observed, and the process accelerated substantially when reporters encoded additional sites (Fig. 11C, 3xmiR-35 and 4xmiR-35). Puzzled by the observation that two copies of miRISC-binding sites could not mediate deadenylation, we considered the arbitrary spacing between our target sites as a possible explanation. In a recent report based on HITS-CLIP as a method to physically map miRISC to mRNAs *in vivo*, the group of Darnell et al. demonstrated that human miRISC protects between 45 and 60 nts on a native mRNA. Because our sites were only spaced by 6 nts, we hypothesized that our 2xmiR-35 and virtually every one of the multi-sites-containing artificial reporters in the literature could accommodate fewer effective miRISCs than intended.



A
= miR-35-42 site
= other miRNA site
2'-O-Me:
-
α-miR-35
α-miR-1
Time (mins)
0 60 180 0 60 180 0 60 180
6xmiR-35 3'UTR
500
1
egl-1 3'UTR
750
500
2
spn-4 3'UTR
1000
750
R05H11.2 3'UTR
750
Y71F9B.8 3'UTR
750
500
3
nhl-2 3'UTR
1000
750
C34H3.1 3'UTR
1000
750
hlh-11 3'UTR
1000
4
B
alg-2 (ok304); mock RNAi
alg-2 (ok304); alg-1 RNAi
Time (mins)
0 60 180 0 60 180
spn-4 3'UTR
R05H11.2 3'UTR
Y71F9B.8 3'UTR

Fig. 10. miRNA-mediated deadenylation is a widespread effect in *C. elegans* embryos.

(A) Time course of endogenous 3'UTR reporter mRNAs from predicted targets of *miR-35-42* in *C. elegans* embryo. Schematic representation of the natural 3'UTRs is depicted on the left. Reporter mRNAs were incubated in the presence or absence of 50 nM 2'-*O*-Me oligonucleotides (either α-*miR-35* or α-*miR-1*). α-*miR-1* 2'-*O*-Me served as a negative control. The natural UTRs fused to a truncated RL ORF are referred to as "short transcripts" in the text. The miRNA sites marked on each UTR representation corresponds to miRNAs that are expressed in *C. elegans* embryos[73]. UTRs are divided into four classes: 1. deadenylated artificial *miR-35* target (6xmiR-35, positive control), 2. deadenylated endogenous *miR-35* target, 3. endogenous UTRs deadenylated by miRNAs, 4. natural UTRs that are not subjected to deadenylation. (B) Time course of Class 3 3'UTR reporters in *alg-2(ok304); gfp (mock) RNAi* or *alg-2(ok304); alg-1 RNAi C. elegans* embryonic extracts. Red bars indicate *miR-35-42* binding sites, blue bars indicate other embryonic miRNA sites[73].

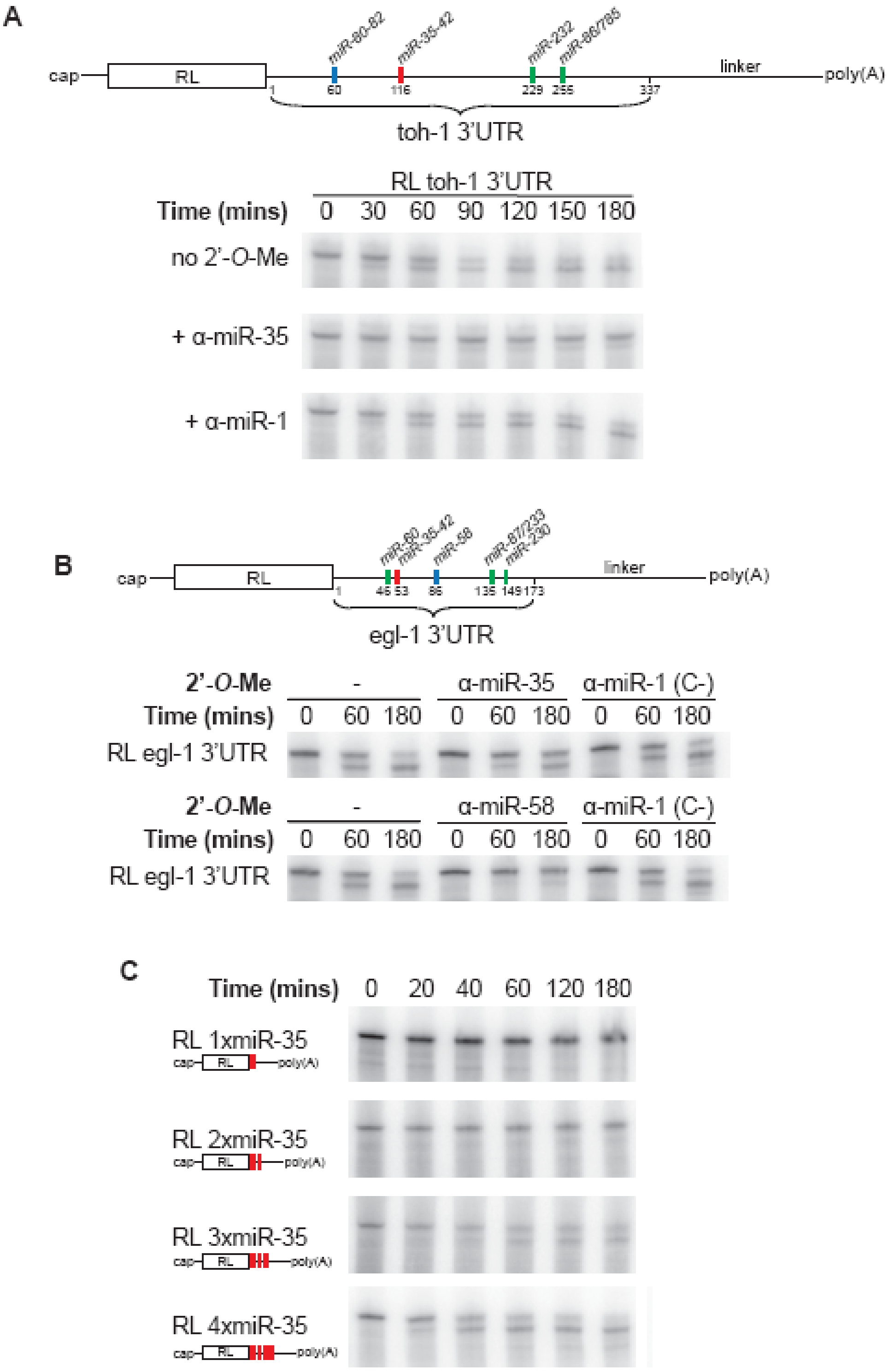

A
cap
RL
miR-80-82
miR-35-42
miR-232
miR-86/785
linker
poly(A)
1
60
116
229
256
337
toh-1 3'UTR
RL toh-1 3'UTR
Time (mins)
0
30
60
90
120
150
180
no 2'-O-Me
+ α-miR-35
+ α-miR-1
B
cap
RL
miR-60
miR-35-42
miR-58
miR-87/233
miR-230
linker
poly(A)
1
46
53
86
135
149
173
egl-1 3'UTR
2'-O-Me
-
α-miR-35
α-miR-1 (C-)
Time (mins)
0
60
180
0
60
180
0
60
180
RL egl-1 3'UTR
2'-O-Me
-
α-miR-58
α-miR-1 (C-)
Time (mins)
0
60
180
0
60
180
0
60
180
RL egl-1 3'UTR
C
Time (mins)
0
20
40
60
120
180
RL 1xmiR-35
cap
RL
poly(A)
RL 2xmiR-35
cap
RL
poly(A)
RL 3xmiR-35
cap
RL
poly(A)
RL 4xmiR-35
cap
RL
poly(A)

Fig. 11. *miR-35* and *miR-58* target *toh-1* and *egl-1* for deadenylation *C. elegans* embryos.

(A and B) Analysis of full-length RL *toh-1* 3’UTR and RL *egl-1* 3’UTR deadenylation in wild-type *C. elegans* embryos. Deadenylation and stability was assayed on A-capped reporters by autoradiography. Reporter mRNAs were incubated in the presence or absence of 50 nM 2’-*O*-Me oligonucleotides (either α-*miR-35*, α-*miR-58*, or α-*miR-1*). α-*miR-1* 2’-*O*-Me served as a negative control. Detailed schematic representation of UTR reporter mRNAs is shown. (C) Deadenylation time course of RL reporter mRNAs with various *miR-35* binding sites. Red bars indicate *miR-35-42* binding sites, blue bars indicate other embryonic miRNA sites, and green bars indicate predicted non-embryonic miRNA sites[73].

CHAPTER 4: DISCUSSION

Despite the extensive research on miRNA biogenesis and action, few studies have provided an understanding of the mechanism underlying miRNA-mediated silencing in the embryonic context. In this study, we established an *in vitro C. elegans* embryonic system that recapitulates miRNA-mediated translational repression and deadenylation. Using this system, we investigated the mechanism and the impact of maternal and zygotic miRNAs on their targets.

4.1 miRNA-mediated repression and deadenylation by maternal and zygotic miRNAs

In eukaryotes, protection of the mRNA at both the 5’ and 3’ ends by the m^{7}GTP cap and poly(A) tail, respectively, allows the mRNA to be more stable and expressed more efficiently in the cell. The 3’ poly(A) tail serves several functions. First, the poly(A) tail increases the efficiency of translation initiation. In conjunction with the 5’ m^{7}GTP cap, the recruitment of initiation factors and their association that bridges the 3’ and 5’ ends of the mRNA results in mRNA circularization, a “closed loop” conformation to facilitate translation initiation and ribosome subunit joining[75, 76]. Second, the poly(A) tail confers mRNA stability. *In vitro* studies have shown reporter mRNAs with a poly(A) tail are more stable than those without[75, 77]. RNA circularization as well as the association of proteins that remain associated with mRNAs during translation also protect mRNAs from degradation by nucleases[78]. Characterization of our *in vitro* system indicates that translation relies heavily on the m^{7}GTP cap and a poly(A) tail and the two features interact synergistically to enhance translation efficiency. RL reporter mRNAs are also stable in the extract. However, when miRNA binding sites corresponding to the maternal *miR-35-42* family and the zygotic *miR-51-56* family were incorporated into the artificial 3’UTR of the RL reporters, translation and the integrity of the mRNA were affected. The length of the poly(A) tail was shortened in a miRNA-dependent and specific manner. In recent years, deadenylation has become a favoured model for miRNA-mediated silencing. miRNAs have been implicated in target deadenylation in many organisms, including mammalian cells, zebrafish, and *D. melanogaster*. We show here, for the first time, translation inhibition and deadenylation of reporter mRNAs by miRNAs *in vitro* in *C. elegans* embryos.

Whether translational repression and mRNA destabilization occur sequentially or as two distinct mechanisms remains controversial. One *in vivo* study proposed that AIN-1 and AIN-2 may coordinate the two processes, as *let-7*-mediated repression and degradation of its targets *lin-41* and *daf-12* was impaired in AIN-1/2 mutants. Following the analysis of other validated *let-7* targets *in vivo*, it was concluded that miRNA-mediated repression frequently, but does not always coincide with mRNA degradation, since some miRNAs did not seem to affect their target mRNA levels[65]. Our study distinctly shows that poly(A) tail removal is a rapid process, beginning within 20 mins of mRNA incubation, and that deadenylation does not result in mRNA degradation, since fully deadenylated mRNAs remain stable. Since translational repression was detected only at about one hour of incubation in our system, we speculate that miRNAs trigger deadenylation of their targets, which in turn leads to translational repression. However, it is to be noted that we cannot exclude the possibility that miRNAs may silence genes via alternative mechanisms. It is possible that other events, independent or coinciding with the deadenylation machinery, may be involved in promoting miRNA-mediated repression. For example, inhibition of translation initiation (independent of target deadenylation) may be significant *in vivo* and could be hidden in our cell-free system where translation takes a long time to initiate.

4.2 A 3’→5’ decay pathway follows miRNA-mediated deadenylation

When aberrant mRNAs are produced, or when an mRNA is targeted for silencing *in vivo*, an mRNA decay pathway can be initiated. Deadenylation is the first step of mRNA decay, which triggers one of two downstream decay pathways: either 1) 5’→3’ decay, which involves the removal of the m^7GTP cap followed by removal of nucleotides by the 5’→3’ exonuclease, XRN-1, or 2) further 3’→5’ decay[79].

A key finding from our deadenylation experiments is the appearance of a stable RNA decay intermediate within two hours of incubation of our reporters. Cloning and subsequent sequencing of this fragment revealed a complete loss of the poly(A) tail for the majority of mRNAs after one hour of incubation. After a three-hour incubation, more than 50% of our clones terminated further upstream, stopping just 3' of the miRNA binding sites. This decay pathway is independent of the m^7GTP cap, indicating that 1) miRNA-mediated deadenylation and decay do not involve the decapping machinery and 2) the decay pathway involves a 3'→5' exonuclease that further removes nucleotides in the 3'UTR until the miRISC sterically hinders and prevents the exonuclease from further 3'→5' destabilization. More work will be needed to define this decay pathway and how it relates to miRNA-mediated deadenylation, specifically whether it is coupled to deadenylation.

4.3 miRNA-mediated deadenylation: a reversible process?

In metazoans, the majority of miRNAs repress translation of targets without inducing mRNA cleavage and degradation. This type of translational regulation offers the possibility for target mRNA reactivation[80]. Two studies, both conducted in mammalian systems, showed specific mRNA targets de-repressed from miRNAs and relocation from P-bodies to the cytoplasm to enter active translation under specific cellular or stress conditions[51, 81]. Under physiological conditions, the cationic amino acid transporter (CAT-1) mRNA is repressed by the liver-specific miRNA, *mir-122*, and repressed mRNA accumulates in P-bodies[51]. Bhattacharyya et al. noticed that under certain stress conditions, such as amino acid deprivation and oxidative stress, CAT-1 mRNA can be relieved from *mir-122*-mediated repression, accompanied by release from P-bodies. In addition, the study also showed that mobilization of the mRNA into the cytoplasm for active translation requires HuR, an RNA-binding protein that is implicated in posttranscriptional regulation by binding to AU-rich elements in its target's 3'UTR[82]. The binding of HuR to CAT-1 mRNA results in enhanced translation and stability, suggesting HuR interferes with the function of miRNAs once the target mRNA is mobilized in the cytoplasm from P-bodies. One important aspect that was not addressed in this study was the status of the poly(A) tail of CAT-1 mRNA.

Another example illustrating reversible regulation of miRNA-mediated silencing is in neurons. The brain-specific miRNA, *miR-134*, is implicated in the regulation of an mRNA encoding for a protein kinase that controls spine development, Limk1[81]. The binding of *miR-134* inhibits the synthesis of new Limk1 protein, thereby restricting the growth of dendritic spines. In response to extracellular stimuli, such as synaptic stimulation, brain-derived neurotrophic factors inactivate *miR-134* inhibition of Limk1 translation, leading to Limk1 translation and dendritic growth. Although the mechanism underlying this reversible regulation is unclear, these findings further describe the dynamic role of miRNAs and miRNP machinery in local and temporal regulation, and their ability to adapt to cellular responses.

4.4 The impact of miRNAs on maternal gene expression in the early embryo

Local temporal and spatial regulation is an important process during animal development. During embryonic development, translation and stability of key mRNAs are tightly controlled to regulate multiple cellular and developmental processes. Early embryonic development is driven by maternal mRNAs. Maternal mRNAs are gene products transcribed by germ cell nuclei in mitosis or early stages of meiosis, and proceed through oogenesis[83]. Upon fertilization, maternal mRNAs become translationally active and play a key role in germ cell development and embryonic polarity[25, 84]. For some genes, zygotic transcription begins at the 4-cell stage in *C. elegans* embryos. However, transcriptional silence is generally maintained until the 100-cell stage, in which embryonic germ cell precursors have been segregated from somatic lineages[85, 86].

Gene regulation in early embryo is governed by an extensive network of post-transcriptional mechanisms. Such regulation allows for rapid and versatile modulation of gene expression at the spatial and temporal level during the period in which zygotic transcription is absent[83]. Maternal mRNAs are tightly regulated by specific factors that assemble onto RNA elements in their 3'UTRs. Multiple *cis*-acting factors can be located on a maternal mRNA's 3' end. Some of these sites may pertain to unique functions, while others may require multiple UTR elements to act in a combinatorial manner[87]. The integrity of these transcripts is regulated throughout early embryonic development. *pal-1* mRNA is a well-known example of a regulated maternal mRNA involved in posterior embryonic development. Maternal *pal-1* mRNA is repressed by two RNA-binding proteins, GLD-1 and MEX-3, until the 4-cell stage, after which the mRNA is de-repressed and localized to the posterior cells[88]. Studies from zebrafish, *D. melanogaster*, and *Xenopus* embryos have identified a clear and sharp transition between the different developmental states. The maternal-to-zygotic transition (MZT) is the transition from maternally-driven to zygotically-driven development. In other words, zygotic transcription is activated and the embryo no longer relies solely on maternally provided transcripts for development[25]. An intriguing study in zebrafish showed that the clearance of many maternal mRNAs is accelerated by *miR-430* via a mechanism involving deadenylation of *miR-430* targets, an embryonic miRNA that is abundantly expressed in zebrafish embryos. Following injection of *miR-430* in Dicer mutant embryos, elimination of *miR-430* maternal mRNA targets was inefficient and severe morphogenic defects in gastrulation and in the brain were observed[89].

Previous studies have shown that the *miR-35-42* family is abundantly expressed in the embryo and is one of the few miRNAs known to be expressed at the 1-cell stage[13, 73]. It is also one of the few miRNAs to date with an embryonic lethal knockout phenotype[73, 90]. Members of the *miR-35-42* family are thought to be deposited maternally, since Lau et al. previously did not detect *miR-35* in mutants depleted of germ cells. Upon re-profiling the expression of *miR-35*, we confirmed that the *miR-35-42* family is indeed contributed maternally, since *miR-35* is present in mutants impaired in male germline development (Fig. 5B). One intriguing aspect that was observed upon sequence alignment of the *miR-35-42* family with the miRNAs involved in MZT in other organisms was the similarity between the *C. elegans miR-35-42* family and the zygotically expressed *D. melanogaster miR-309* (data not shown). The two seeds only differed by one nucleotide located at position 5 of the seed. This observation could suggest that this miRNA family may have a conserved function in maternal mRNA regulation and early development. However, the fact that the *miR-35-42* family is loaded maternally, and that we observed stable deadenylated reporters argues in favor of a divergence in function. Another intriguing observation, this one based on the miRISC pulldown, is that a substantial amount of ALG-1 and ALG-2 is associated with *miR-35-42*. In other words, a large quantity of embryonic miRISC is programmed by this single family. This finding highlights the significance of the *miR-35-42* family in early development, and in general, the profound impact of maternal miRNAs in embryonic development.

4.5 miRNA-mediated deadenylation is prevalent in *C. elegans* embryos

Our mid-scale analysis of UTR targets identified multiple miRNA targets and unveiled the complexity of the collaboration between maternal and zygotic miRNAs. Of the 13 endogenous 3'UTRs that were screened for mRNA deadenylation and decay, 7/13 UTRs were deadenylated. In all cases, this effect was miRNA-dependent, since deadenylation was impaired in the ALG-1/2 depleted extracts. On its own, this observation is fascinating, as it reveals a prevalent impact of miRNAs on the poly(A) tail of embryonic mRNAs. However, *miR-35-42* did not seem sufficient in triggering deadenylation of some of the UTRs, since a sequence-specific inhibitor did not slow down deadenylation. A comparison of the screened UTRs to the miRNA reads obtained from early *C. elegans* embryos published by Stoeckius et al. suggested that other embryonically expressed miRNAs could be responsible for the regulation of these UTRs. Thus, multiple miRNA families appear to coordinate pressure on mRNAs and define spatial and/or temporal regulation in the embryo.

On the other hand, two of the screened targets, *to*llis*h-1* (*toh-1*) and *eg*g-*l*aying defective-*1* (*egl-1*) could be directly linked to the *miR-35-42* family. *toh-1* belongs to the tolloid and bone morphogenic protein (tld/BMP-1) family of proteins. The tld/BMP-1 family encode for astacin zinc-binding metalloproteases, a family of proteases whose function include activation of growth factors and processing of extracellular proteins[91]. For example, studies in *D. melanogaster* revealed that *toh-1* is indeed involved in the activation of extracellular growth factors by genetic interactions with a member of the *t*ransforming *g*rowth *f*actor-*beta* (TGF-β)-like family, regulating various developmental processes, such as the establishment of the dorsoventral axis of *D. melanogaster* embryos[92-94]. Several studies from different organisms have also shown *toh-1* is involved in embryonic development[92-96]. *C. elegans* possess two *toh* members, *toh-1* and *toh-2*, but they have yet to be characterized[97]. In *C. elegans*, the TGF-β signaling pathway is involved in processes that include body size maintenance, tail morphogenesis, and dauer larva formation[98, 99]. One possible function for the *miR-35-42* family would be to temporarily silence *toh-1* mRNA from early development until the TGF-signaling pathway needs to be activated for cell differentiation and morphogenic processes in later developmental stages. TargetScan prediction algorithm also revealed another miRNA site on *toh-1*, a site complementary to the seed corresponding to the *miR-80-82* family. According to Stoeckius et al. *miR-81* is expressed in 1-cell stage embryos. Although we did not examine whether *miR-81* is on its own required for *toh-1* deadenylation, it is likely that *miR-81* collaborates with the *miR-35-42* family in the regulation of *toh-1* early in development.

egl-1 encodes an activator of the programmed cell death pathway. The protein contains a Bcl-2 homology region 3 (BH3), a domain found in pro-apototic factors. *egl-1* is transcriptionally repressed by TRA-1, a Zn finger domain DNA-binding protein that binds to *egl-1* promoter and represses *egl-1* expression in hermaphrodite-specific neurons (HSNs). This process is important for female sex determination during development[100]. However, *egl-1* activity is not only regulated at the level of HSNs, since *egl-1* gene is transcriptionally active specifically in cells that are destined to die during development[101]. The current model for cell death specification is that in the 959 cells destined to survive during development, EGL-1 activity is low or absent and that in the remaining 131 cells destined to die, EGL-1 activity is high, activating the apoptotic pathway and causing cells to commit to the cell death fate[102]. In a recent study, *egl-1* was reported to be a target of the *miR-35-42* family, but their validation was based on indirect observations[103]. This report was based on two observations: 1) *egl-1* mRNA was co-immunoprecipitated with AIN-2 in embryos and 2) a transgenic reporter encoding *egl-1* UTR was repressed when co-expressed with a construct overexpressing the *miR-35-42* family in ectopic tissues. The mechanism of miRNA-mediated silencing and the biological impact of miRNAs regulating *egl-1* expression were not addressed. Our screen identified *egl-1* as a deadenylated target of both the *miR-35-42* family and *miR-58*, indicating that both miRNAs can contribute to the silencing of *egl-1*. Our analysis demonstrates that the maternal *miR-35-42* family is abundantly expressed in early embryos, and that its expression is dramatically decreased from larvae to adults. Conversely, *miR-58* is expressed throughout development, with a peak expression in L1 larvae. Since sequence analysis of *egl-1*'s 3'UTR reveals a binding site for both *miR-35-42* and *miR-58*, we hypothesize that *miR-35-42* mainly regulates *egl-1* in early embryos, and *miR-58* takes over and becomes the primary regulator of *egl-1* following the activation of zygotic transcription, providing a finely tuned cell survival signal.

4.6 Spacing requirements for miRISC:mRNA interaction

In vivo and *in vitro* studies often use reporters that encode multiple miRNA binding sites to study efficient miRNA-mediated repression. Our results show that artificial reporters with at least three complementary miRNA sites are targeted for deadenylation. However, in the case of the endogenous UTR reporters, *toh-1* and *egl-1*, two embryonic miRNAs sites are required to trigger deadenylation. In *egl-1* 3'UTR, for example, *miR-35-42* and *miR-58* binding sites are separated by 29 nucleotides. When either *miR-35* or *miR-58* was blocked, deadenylation was prevented (Fig. 11). This suggests that two miRISCs are required, and that there is a minimum spacing required for miRNA-mediated silencing. During the course of our studies, it was reported that miRISCs protect a sequence of 45-60 nucleotides footprint on an mRNA target[104]. Together with our findings, this suggests miRNA sites need to be correctly spaced on artificial reporters to prevent steric hindrance between binding sites so that they can act cooperatively and synergistically[39]. The miRNA sites on our reporters were closely spaced, with approximately six nucleotides separating each miRNA site. Based on Chi et al.'s report and our results on *egl-1*, it is likely that our RL 3xmiR-35 reporter contains only two functional *miR-35* sites, while our RL 2xmiR-35 reporter, which is not deadenylated *in vitro*, contains in effect only one functional *miR-35* site. Our data, however, does not eliminate the possibility of other factors contributing to miRNA-mediated deadenylation. Since 3'UTRs frequently encode regulatory elements to regulate the expression and stability of mRNAs, it is possible that other non-miRNA elements in the 3'UTR work alongside the miRISC.

4.7 A model for miRNA-mediated deadenylation and RNA decay in embryos

Based on our findings, we propose the following model (Fig. 12): RNAs that are not immediately necessary in the embryos are temporarily silenced by specific miRNAs that guide RISC to their 3'UTRs. Deadenylation factors are then recruited to silence the mRNA. Our results suggest that the fully deadenylated reporters are stable with time rather than subjected to degradation. When expression is required, specific cues in the 3'UTR will signal the readenylation of the mRNA by recruiting a poly(A) polymerase (PAP). If the stable deadenylated intermediate is not readenylated, a 3'→5' exonuclease will further destabilize the mRNA to consolidate silencing of the gene. miRNA-mediated deadenylation as a reversible process is of particular relevance to maternal mRNAs targeted by maternal miRNAs, such as the *miR-35-42* family. One informative prediction is that some of the *miR-35-42* targets are stored in a deadenylated state in the early embryo. More work will be needed to test this prediction and address how and when readenylation and translation reactivation are initiated.

In summary, our findings demonstrate that deadenylation is a key mechanism in miRNA-mediated silencing in early *C. elegans* embryo. In a screen to validate targets of the maternal *miR-35-42* family, we showed that more than half of the predicted targets were subjected to miRNA-mediated deadenylation, indicating this phenomenon is widespread in *C. elegans* embryos. We identified *toh-1* as a target of *miR-35-42* and *egl-1* as targets of both *miR-35-42* and zygotic *miR-58*. Our results also implicated a 3'→5' decay intermediate step that may a serve a purpose in consolidating silencing of genes not required for early embryonic development. Taken together, our studies provide an understanding of miRNA-mediated deadenylation and destabilization and its impact on early animal development.

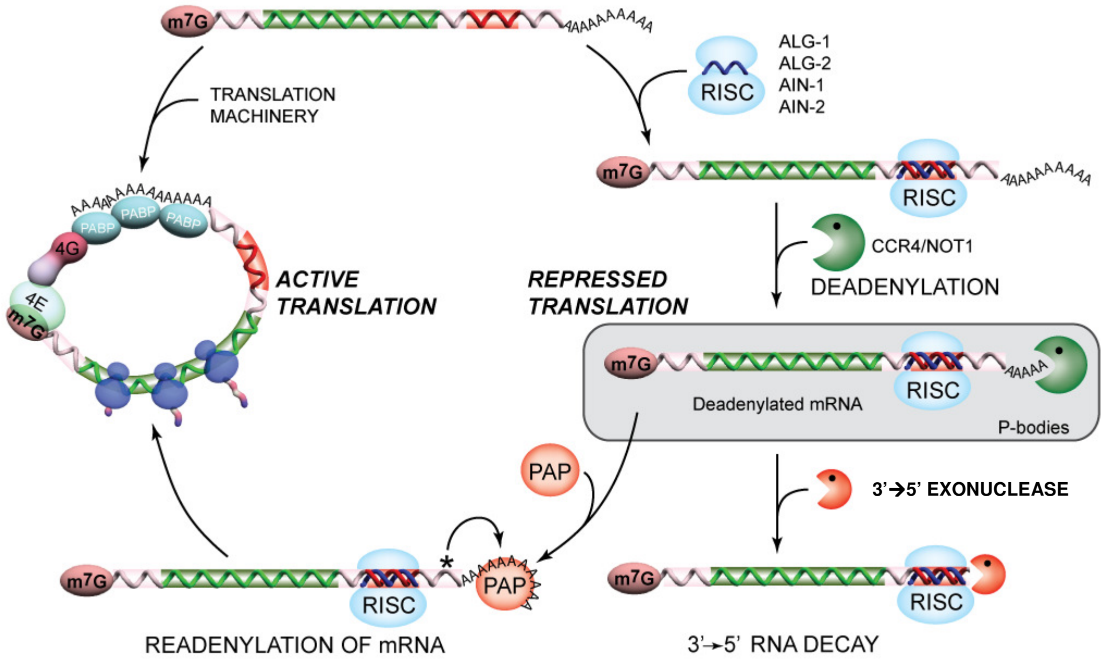


Fig. 12. Model for miRNA-mediated deadenylation and RNA decay in embryos.

Target-specific miRNA guides the RISC to the 3’ UTR of the gene. Deadenylation factors are then recruited to silence the mRNA that is not actively translated in the embryo. Deadenylation of the target mRNA is thought to occur in P-bodies, the sites for mRNA deadenylation, degradation, and temporarily silenced mRNAs. When translation needs to be restored, specific cues will act on other regions (denoted by *) in the 3’UTR, signaling for the readenylation of the mRNA by recruiting a poly(A) polymerase (PAP). If the stable deadenylated intermediate is not readenylated, a 3’ → 5’ exonuclease will further destabilize the mRNA, consolidating silencing of the gene.

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