Mechanisms of microRNA mediated gene silencing in *C. elegans*

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Professor Dr. Eberhard Parlow

(Dekan)

It is a source of embarrassment to me that, after having argued at length that protein synthesis is regulated by mechanisms which operate in the cytoplasm of the cell, I am unable to provide any serious body of experimental evidence concerning the precise chemical nature of this regulation.

Sir Henry Harris
Nucleus and cytoplasm. Oxford: Clarendon Press (1974).

If it don't fit, don't force it
Just relax and let it go
Just 'cause that's how you want it
Doesn't mean it will be so

If it don't fit don't force it
Composed by Larry Farrow and Carolyn Johns, interpreted by Kellee Patterson (1978).

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1. Summary

microRNAs (miRNAs) are a large family of small non-coding RNAs, which post-transcriptionally repress numerous genes; a type of regulation which is important for countless physiological processes of multicellular organisms in health and disease.

miRNAs are genomically encoded and transcribed as long precursors, which undergo a refined and tightly regulated maturation process giving rise to ~22 nucleotide-long RNAs. These small RNAs function as part of an RNA-protein complex termed miRNA induced silencing complex (miRISC). miRNAs, and by extension miRISCs, typically bind partially complementary elements in the 3' untranslated region (UTR) of target messenger RNAs (mRNAs), which become consequently repressed. A large set of studies clearly indicates that miRISC-mediated repression is achieved in metazoans by a variable combination of target mRNA degradation and translational repression. However, the phase of translation that is inhibited is a controversial subject. Various models have been proposed, based mostly on the study of artificial target reporters, which support either an initiation or a post-initiation block model. Argonaute proteins are core component of the miRISC and directly bind miRNAs. The GW182 protein, another miRISC component, has recently emerged as an essential mediator of miRNA-mediated repression action. However, its precise molecular function is still unclear.

The work presented here aimed at understanding the *in vivo* mechanistic aspects of miRNA-mediated repression in more details, using the nematode *Caenorhabditis elegans* as a model organism and focusing on the well characterized *let-7* miRNA. Initial results from a large-scale genetic screen revealed a strong genetic interaction between *let-*7 and various translation initiation factors, leading me to propose that *C. elegans* miRNAs inhibit the initiation of translation on their target mRNAs. Additional genetic experiments uncovered an unsuspected widespread genetic interaction between *let-7* and the translation machinery and suggested that *let-7* might function by inhibiting the eukaryotic translation initiation factor 3 (eIF3) activity. Biochemical experiments demonstrated that a large set of *C. elegans* miRNA targets are translationally repressed at the initiation step, sometimes in combination with mRNA degradation, and that the *C. elegans* GW182 homologs AIN-1 and AIN-2 are essential for these mechanisms. Additional unpublished data revealed that translational repression is

specifically mediated by AIN-1, whereas depletion of both AIN-1 and AIN-2 is necessary to prevent miRNA target degradation.

Collectively, these results show that *C. elegans* miRNAs employ at least two mechanisms *in vivo*, i.e. target degradation and inhibition of translation initiation, which are likely to be independent pathways.

2. Introduction

2.1. Regulatory noncoding RNAs

Molecular biology has been traditionally centered on the view that RNA molecules merely represent message transporters between the DNA encoded genetic information and functionally active proteins. In higher eukaryotes, this view has been challenged based on several observations (reviewed in (Mattick 2003)). First, in higher organisms the number of protein-coding genes is not only lower than expected, but also fails to reflect biological complexity: humans contain only about 30% more protein-coding genes than the simple nematode *Caenorhabditis elegans* (approximately 25‘000 and 19‘000, respectively). At the same time, the ratio between noncoding and coding sequences of the genome increases from 1,3 in *C. elegans* to 47 in humans (Frith et al. 2005). Second, although the human euchromatic genome is composed of only 1.2% percent of protein-coding genes (Consortium 2004), more than 90% of it is likely to be transcribed on one or both strands (Birney et al. 2007). Indeed, ~98% of the transcriptional output of mammalian genomes is composed of noncoding transcripts (Mattick 2003). Third, an increasing number of noncoding RNAs has been shown to be functionally active, regulating processes as diverse and important as chromosome structural organization and messenger RNAs (mRNAs) translation (reviewed in (Amaral et al. 2008)). Fourth, these noncoding RNAs appear to be developmentally regulated (Dinger et al. 2008) and in some cases, associated with diseases (reviewed in (Szymański and Barciszewski 2008)). Finally, promoter regions of noncoding genes do not only contain binding sites for common transcription factors (Cawley et al. 2004) but are also generally more conserved than the ones of protein-coding genes (Carninci et al. 2005). These accumulating evidences point to a much broader role of RNA molecules than previously acknowledged in the function, and more importantly, the regulation of cellular processes. Although there is still some debate about how much noise this high level of noncoding transcription represents, it has been proposed to have allowed higher eukaryotes to integrate the network of information needed to develop highly complex biological processes (Mattick 2001). In parallel to the “RNA world” hypothesis, which proposes that DNA-based organisms originated from a cellular and pre-cellular RNA-based life, the idea of a “modern RNA world” has been proposed, postulating that RNA is a more optimal

material than protein for certain functions and is therefore also employed for recently evolved and sophisticated tasks (Eddy 2001).

Regulatory noncoding transcripts are generally referred to as long or small noncoding RNAs, i.e. longer and shorter than approximately 300 nucleotide (nt) long. Rather than an objective size threshold, this classification reflects the fact that the former are much less characterized than the latter, which have only their relative small size in common. The importance of long noncoding RNAs is just starting to be appreciated and few paradigms of regulation mediated by these are appearing (reviewed in (Wilusz et al. 2009)). In general, such RNAs can modulate the transcription of downstream genes, either in *trans* or in *cis*, influence the processing of transcripts by base-pairing mechanisms, modify the activity of protein-binding partners, or serve as precursors of small RNA molecules. One outstanding example, due to its physiological importance, is X-chromosome inactivation (XCI). XCI is initiated by the recruitment of the Polycomb complex by the *RepA* noncoding RNA expressed from the *Xist* gene, which in turn induces repressive chromatin modifications on the chromosome to be inactivated (Zhao et al. 2008). It is likely that more regulatory mechanisms relying on long noncoding RNAs are yet to be discovered.

Regarding small noncoding RNAs, much attention has been brought to the so-called small silencing RNAs. Since their discovery in the early 1990s, they were shown to play an important role in regulating gene expression. The best understood classes of small silencing RNAs are the small interfering RNAs (siRNAs), microRNAs (miRNAs), and the more recently identified Piwi-interacting RNAs (piRNAs) (reviewed in (Ghildiyal and Zamore 2009)). siRNAs, miRNAs, and piRNAs share some similarities: they range between 20 to 30 nucleotides in size, they associate with members of the Argonaute (Ago) protein family, and typically induce repression of target genes via a base-pairing mechanism. Beside these commonalities, these RNAs follow different biogenesis pathways, interact with distinct sets of factors, and are implicated in different regulation mechanisms.

The effects of small silencing RNAs were first documented in plants. The use of antisense transgenes of *chalcone synthase* (*CHS*), which encodes for an enzyme involved in flower pigmentation, resulted in a decrease, rather than an increase, of pigmentation intensity, due to a strong downregulation of endogenous *CHS* mRNA levels (van der Krol et al. 1988; Napoli et al.

1990). The exact mechanism involved remained elusive until further studies conducted in plants, *Drosophila melanogaster*, and *C. elegans* revealed that gene silencing can be triggered by long double stranded RNAs (dsRNAs), which are processed by Dicer, a type III endoribonuclease, into siRNAs (Fire et al. 1998; Hamilton and Baulcombe 1999; Hammond et al. 2000; Zamore et al. 2000; Bernstein et al. 2001). In brief, siRNAs function as part of the Ago-containing RNA induced silencing complex (RISC), which is recruited to target transcripts by perfect antisense base-pairing. Ago proteins direct the endonucleolytic cleavage (slicing) of targets and the resulting RNA fragments are rapidly degraded (reviewed in (Carthew and Sontheimer 2009)). This process, i.e. the conversion of long dsRNAs into siRNAs followed by post-transcriptional gene silencing (PTGS), has been termed RNA interference (RNAi). The discovery of RNAi had a deep impact on our understanding of molecular biology, both as tool and as a natural phenomenon (Novina and Sharp 2004). Exogenous long dsRNAs, from viruses or transgenes were originally thought to be the unique source of siRNAs, and therefore RNAi was essentially seen as a defense mechanism against foreign nucleic acids. It was later discovered that siRNAs can also be produced from endogenous triggers in plants and animals and control the expression of endogenous genes and transposons (reviewed in (Vazquez 2006; Okamura and Lai 2008)). In addition to playing a role in PTGS, siRNAs are also involved in heterochromatin formation in plants, fission yeasts, flies and nematodes (reviewed in (Buhler and Moazed 2007)), highlighting the broad action range of this type of small RNAs.

piRNAs differ significantly from siRNAs and miRNAs: they are produced by a poorly understood, but Dicer-independent, mechanism from single stranded precursors (reviewed in (Klattenhoff and Theurkauf 2008)). They interact with the Piwi clade of Ago proteins, whereas siRNAs and miRNAs do so with the Argonaute clade (see below). Their expression is restricted to the germline, where they seem to silence retrotransposons and thereby protect germline DNA integrity (Klattenhoff and Theurkauf 2008).

miRNAs represent the third well defined class of small silencing RNAs. miRNA biology is relatively well understood, due to extensive research efforts over the last 10 years. Yet, some aspects still remain unclear, especially about their precise mode-of-action. In the following sections, after a brief introduction about miRNA discovery, I present our current understanding of miRNA biogenesis, mode-of-action, and biological functions.

2.2. microRNA biology

2.2.1. The discovery of microRNAs

C. elegans develops to adulthood through four larval stages (L1 to L4), each separated by a molt. During this development, cell division patterns are essentially invariant among individuals, both in time and space, and have been extensively mapped (Sulston and Horvitz 1977; Kimble and Hirsh 1979). This makes it possible to study mutants in which these patterns are altered. The locus of the first known miRNA, *lineage-4* (*lin-4*), was initially described in 1981 for this reason (Chalfie et al. 1981). In *lin-4* animals, certain cells reiterate L1 parental division pattern during the L2 and L3 stages. Additionally, and probably consequently, *lin-4* mutants exhibit a delayed developmental timing and fail to form the adult cuticle, termed alae. (Chalfie et al. 1981). During the following years, numerous genetic and biochemical studies from the Ambros and Ruvkun laboratories helped understanding the role of *lin-4* (reviewed in (Neilson and Sharp 2008)). It appeared that *lin-14* loss-of-function (LOF) animals have a phenotype opposite to *lin-4*. Cells which adopt a retarded phenotype in *lin-4* animals show a precocious development in *lin-14* LOF, that is they adopt a L2 fate at the L1 stage already. Conversely, *lin-14* gain-of-function mutations induce a similar phenotype as *lin-4* mutations. Further analyses showed that *lin-4* is epistatic to *lin-14*. It also became clear that elements in 3' untranslated region (UTR) of *lin-14* mRNA were necessary and sufficient to allow *lin-4* to repress *lin-14* expression from the L2 stage onward. Nevertheless it was not before 1993 that the molecular identity of *lin-4* was elucidated: a ~22 nucleotide-long RNA with partial antisense complementarity to elements of the *lin-14* 3'UTR (Lee et al. 1993). The *lin-4* - *lin-14* interaction established the paradigm of miRNA action: a small RNA represses the expression of a target mRNA by binding imperfect complementary elements in its 3'UTR. However, before becoming a classical example, this regulation was considered only as a curiosity: one small non-conserved RNA regulating one mRNA in *C. elegans*.

This all changed 7 years later with the discovery of a second *C. elegans* miRNA: *lethality-7* (*let-7*) (Reinhart et al. 2000). *let-7,* which, as its name implies, is required for worm viability, was identified by a genetic screen devised to find new heterochronic genes, i.e. genes which, like *lin-4* and *lin-14,* regulate developmental timing. Reinhart and co-workers showed that *let-7* expression starts at the L3 stage and represses *lin-41* expression, which in turn allows

the *lin-29* transcription factor to be expressed and worms to properly reach the adult stage. Because similarly to *lin-4*, *let-7* was shown to be a heterochronic gene itself and to regulate other members of this pathway, these RNAs were termed small temporal RNAs (stRNAs). However, this denomination was replaced by "microRNA" when it became clear later that not all members of this family regulate developmental timing.

Realizing that *let-7* was not restricted to *C. elegans*, but highly conserved among bilaterians, with 100% homology between *C. elegans* and *H. sapiens* (Pasquinelli et al. 2000), gave its momentum to the miRNA field. Three studies, simultaneously published in *Science* in 2001, reported the identification of numerous small RNA similar to *lin-4* and *let-7* in *D. melanogaster* and *C. elegans* and suggested to name this class of RNA "microRNA" (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). At the same time, few miRNAs were also identified in *Arabidopsis thaliana* (Reinhart et al. 2002).

Since 2001, the number of known miRNA did not stop to grow. The current miRBase release (13.0) refers almost 10'000 miRNAs among which 187 in *A. thaliana*, 152 in *D. melanogaster*, 155 in *C. elegans*, and 706 in *H. Sapiens* (Griffiths-Jones et al. 2008). Beside plants and metazoans, miRNAs have also been detected in some viruses (reviewed in (Cullen 2009)) and in the unicellular algae *Chlamydomonas reinhardtii* (Molnár et al. 2007; Zhao et al. 2007), suggesting that this type of RNA is evolutionary ancient.

Altogether, miRNAs represent today a large family of molecules regulating many genes relevant to important cellular and physiological processes (see section 2.2.5.). They are defined by a few common features which set them apart from siRNAs or piRNAs. They do not derive from long dsRNA molecules like siRNAs, but from hairpin-folded single stranded precursors, which undergo a specific maturation process (see section 2.2.2. and 2.2.3.) and, unlike piRNAs, require Dicer for their biogenesis. Additionally, at least in animals, they normally do not induce target endonucleolytic cleavage like siRNAs, but rather mediate target repression via a combination of translation inhibition and mRNA destabilization (see section 2.2.4.). This difference is thought to result mainly from the nature of the binding between miRNAs and their targets. Animal miRNAs, except for a few counterexamples (Yekta et al. 2004; Davis et al. 2005) and contrary to plants miRNAs, bind target RNAs with limited complementarity, mostly restricted to the 5' region of the microRNA, the so-called "seed" region (reviewed in (Bartel

2009)). Another striking feature of miRNAs is that, although artificial binding sites placed in mRNA 5'UTRs or open reading frames (ORFs) seem functional (Saxena et al. 2003; Kloosterman et al. 2004; Lytle et al. 2007), endogenous binding sites are almost exclusively located in the 3'UTR of target mRNAs. One notable exception is the human miR-10a, which binds to mRNA 5'UTRs. However these interactions do not silence, but rather seem to stimulate target expression (Orom et al. 2008).

Similarly to siRNAs and piRNAs, miRNAs function in a complex with Ago proteins (reviewed in (Hutvagner and Simard 2008)). Ago proteins are essential for the function of small silencing RNAs and define the core of RISCs. They contain two RNA-binding domains: PAZ (PIWI/Argonaute/Zwille) and Piwi, which interact with the 3' and 5' ends of small silencing RNAs. Agos are present in bacteria, archea and eukaryotes and have undergone a high degree of gene duplication, especially in plants and metazoans. There are 8 Ago genes in humans, 5 in *D. melanogaster*, 10 in *A. thaliana* and 27 in *C. elegans*. Based on phylogenetic analysis, these genes can be classified in three groups: the Argonaute-like proteins (similar to *A. thaliana* AGO1), the Piwi-like proteins (similar to *D. melanogaster* PIWI), and the *C. elegans* specific group 3 Argonautes (Hutvagner and Simard 2008). Some Agos are specialized for one type of small silencing RNA. For example, piRNAs interact exclusively with Piwi-like Agos. Similarly, two out of the 27 *C. elegans* Agos are specific for miRNAs: ALG-1 and ALG-2 (Grishok et al. 2001).

2.2.2. Biogenesis and post-transcriptional regulation of microRNAs

miRNA biogenesis is a multistep process. The production of ~22 nt-long miRNAs starts with the transcription of a primary-miRNA (pri-miRNA) of hundreds, sometimes thousands of nucleotides. Pri-miRNAs are first cleaved in the nucleus by the Drosha-Pasha complex to produce ~70 nt-long precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported in the cytoplasm where their cleavage by Dicer produces mature miRNAs able to associate with the RISC complex, termed miRISC in this case, and direct target transcript repression (this pathway is presented in more details in the review included in section 2.2.3.). Initially, miRNA biogenesis was thought to be a rather passive process. It was assumed that pri-miRNAs are directly and automatically converted into active miRNAs. However, an ever-increasing number of evidence shows that miRNA maturation is tightly regulated. This aspect of miRNA biology is reviewed in detail below (Ding et al. 2009). Since the publication of this review, two facets of miRNA life-cycle regulation have seen significant developments. These are the regulation of *let-7* processing by the pluripotency factor LIN28 and the discovery of an active miRNA turnover mechanism in *C. elegans*.

The *Lin28* gene, in addition to being a *let*-7 target itself, encodes an RNA-binding protein which regulates the processing of *let-7* family members, i.e. *let-7* and other miRNAs with identical 5' sequence. It has been proposed that Lin28 directly competes with Drosha and Dicer for the binding of the pri-miRNA and pre-miRNA intermediates, respectively (Newman et al. 2008; Rybak et al. 2008; Viswanathan et al. 2008). A subsequent study suggested that LIN28 induces pre-let-7 3' uridylation, which blocks its processing and directs it for degradation (Heo et al. 2008). Two recent studies in human cell lines and mouse embryonic stem cells substantiated this model by identifying the terminal uridyl transferase 4 (TUT4) as the enzyme involved in this process (Hagan et al. 2009; Heo et al. 2009). Moreover, a similar mechanism has been found to occur in *C. elegans* via the poly(U) polymerase PUP-2 (Lehrbach et al. 2009). Uridylation of miRNA intermediates is therefore a conserved mechanism and a potentially important way of regulating miRNA maturation. Interestingly, the addition of a 3'-terminal adenine to the mature miR-122 by the poly(A) polymerase GLD2 was recently shown to stabilize this miRNA in human hepatocytes (Katoh et al. 2009). These studies indicate that

sequence modification of miRNAs or miRNA precursors might be a widely employed mechanism to selectively regulate their processing and stability.

It is conceivable that mature miRNA level, and thereby activity, might also be regulated by an active degradation mechanism. However, besides the identification in plants of SDN1, a "small RNA degrading nuclease" able to degrade miRNAs *in vitro* (Ramachandran and Chen 2008), there was so far no evidence for such a mechanism. Recently, Chatterjee and Grosshans showed that *C. elegans* mature miRNA levels are modulated by the 5' to 3' exonuclease XRN-2 (Chatterjee and Grosshans 2009). XRN-2 depletion was shown to elevate the levels of several miRNAs and to reinforce *let*-7 target repression *in vivo*. Additionally, *in vitro* experiments using larval lysates suggested that miRNAs are first released from Ago proteins before being degraded by XRN-2. Interestingly, this release step can be blocked by the addition of target RNA molecules to the lysates. This suggests that the presence of miRNA targets might indirectly regulate the level of their cognate miRNAs and implies that miRNA clearance might be triggered by the disappearance of their targets.

Considering the broad effects of miRNAs on many biological processes (see section 2.2.5.), it is not surprising that their activity has to be precisely controlled. Recent data now show that this control can take place at every step of the miRNA life-cycle, from pri-miRNA transcription to mature miRNA clearance.

2.2.3. Publication: “Regulating the regulators: mechanisms controlling the maturation of microRNAs”

Review

Regulating the regulators: mechanisms controlling the maturation of microRNAs

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MicroRNAs (miRNAs) are small noncoding RNAs that control diverse cellular and developmental events through repression of large sets of target mRNAs. Regulated transcription of the genes encoding miRNAs by RNA polymerase II promotes specific expression patterns of individual miRNAs. However, recent studies have established that substantial regulation of mature miRNA accumulation also occurs after transcription. Here, we review the mechanisms of such post-transcriptional regulation, with a particular focus on examples where molecular mechanisms or physiological principles are beginning to emerge. Elucidating these mechanisms will increase our understanding of gene regulation and provide new insights into causes of miRNA misexpression in diseases such as cancer.

Introduction

MicroRNAs (miRNAs) are a large class of genomically encoded 22 nucleotide (nt)-long RNAs that regulate target mRNAs in plants and animals through an antisense mechanism [1,2]. The first miRNA, *lin-4*, was identified in a genetic screen for mutations causing abnormal temporal regulation of cell differentiation in the nematode *Caenorhabditis elegans* [3], and it was initially considered to be unique to this organism. However, in the 15 years since, small RNA cloning and computational sequence analysis have tremendously expanded the number of known miRNAs and of organisms in which they occur; currently, there are 695 miRNAs known in humans, 488 in mouse, 152 in the fly *Drosophila melanogaster* and 154 in *C. elegans* (miRBase 12.0, http://microrna.sanger.ac.uk/sequences/; [4]). miRNAs thus outnumber even large classes of regulatory proteins such as kinases, providing a first indication of the pervasiveness of gene regulation by miRNAs. Indeed, each miRNA has several, possibly dozens or even hundreds of targets (e.g. Refs [1,5,6]), which it recognizes by binding to partially complementary sequences in the 3′ untranslated regions (3′UTRs) of the target mRNA [2]. Binding of the target results in repression of the target mRNA through mechanisms that have not been fully elucidated but seem to involve translational repression, deadenylation and degradation of the target mRNAs [2].

Consistent with their abundance, miRNAs are involved in various developmental and cellular processes, regulating, for instance, stem cell fates, apoptosis and metabolism, and miRNA dysregulation has been implicated in various diseases, particularly cancers [1,7]. Accordingly, much effort has been put into developing robust profiling methods for miRNA expression patterns (Box 1) as experimental tools for the biologist and potential diagnostic tools for the clinician.

Although mature miRNAs are only ~22 nt long, their biogenesis is a complex affair; transcription of a primary miRNA (pri-miRNA) of several hundred or thousands of nucleotides in length is followed by two processing steps mediated by the two nucleases Drosha and Dicer, respectively, and then loading of the mature miRNA into a functional protein complex containing an argonaute (AGO) protein at its core (Figure 1; [7,8]). We are now beginning to learn that the miRNA maturation events are highly regulated, subjecting these small post-transcriptional regulators of gene expression themselves to extensive post-transcriptional control. We will discuss here recent work that has begun to elucidate some of the regulatory mechanisms, which can be specific for individual or a few miRNAs or can affect miRNA expression more globally. We will point out examples where physiological consequences of such regulation are beginning to emerge. Given the numerous examples where aberrant miRNA expression contributes to pathologies, we predict that these regulatory principles will provide important insights into molecular mechanisms of disease and might ultimately yield novel targets for therapeutic intervention.

miRNA biogenesis: the main players

Transcription of the pri-miRNA is the first step along a complex pathway that generates an active RNA-induced silencing complex (RISC) loaded with a mature miRNA – the so-called miRISC (Figure 1). Although a few miRNAs seem to be transcribed by RNA polymerase III [9], the bulk of pri-miRNAs is transcribed by RNA polymerase II, the same enzyme that produces protein-coding mRNAs and is accordingly subject to similar intricate means of transcriptional regulation [7,10]. Indeed, a large fraction of vertebrate miRNAs is encoded in the introns of protein-coding host genes, with whom they are thus co-transcribed. However, the promoters of 'intergenic' pri-miRNAs – that is, those that are encoded outside protein-coding host genes – are also subject to input from numerous transcriptional

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Box 1. MicroRNA expression analysis: opportunities and challenges

To understand the function of miRNAs in biology and exploit their utility as disease markers, robust and sensitive miRNA profiling technologies are required. Recent years have indeed seen a move from research-driven to application-oriented platforms comprising oligonucleotide microarrays, PCR or high-throughput cloning and sequencing approaches [78]. Nonetheless, considerable disagreement frequently exists with respect to miRNA expression patterns for a specific tissue or cell-type, which at least in part could be due to technical differences among platforms [79].

As for mRNA expression profiling, variations at any stage in the process, from sample collection and storage to RNA isolation, detection and data analysis, can influence the quality and accuracy of the final results. Additional challenges are imposed by the low abundance of miRNAs (~0.01% of the total RNA mass) and their small size, which leaves little flexibility for label attachment and probe design. Accordingly, achieving comparable melting temperatures for all miRNA–probe hybrids, which is essential to avoid significant numbers of false negatives and false positives, requires substantial effort [78]. Furthermore, although miRNA microarrays might preferentially detect mature miRNAs [80], this specificity might be insufficient when precursors accumulate to significant levels. The stem–loop TaqMan reverse transcription (RT)–PCR reagents [78] are one example of alternative tools with increased specificity for mature miRNAs, but global expression profiling using this system is cost-intensive and does not permit detection of the precursor forms.

Apart from technical problems associated with measuring miRNA expression, the subsequent analysis of miRNA expression data is nontrivial, mainly due to the lack of standardized normalization and scaling methods [81]. Consequently, efforts to standardize miRNA profiling protocols and establish thoroughly characterized reference sets are of paramount importance, and validation of putative expression differences established by microarray-based approaches by quantitative (q)RT–PCR or northern blotting remains advisable.

The total number of miRNAs keeps increasing, which makes it difficult to compare miRNA expression datasets that have been generated by different platforms based on different miRBase releases [4]. Finally, recent reports using deep-sequencing technology suggest that certain miRNAs can occur in more than one variant, dependent on the cell- or tissue-type [82]. Sequences differ primarily at the 3′-end but also at the 5′-end of the molecule. Although the biological significance of these observations is unclear, reliable, high-throughput discrimination is unlikely to be feasible with available hybridization-based technology and might require deep-sequencing instead. Regardless of the technical strength or weakness of individual profiling technologies, *in situ* hybridization approaches [83] are crucial for establishing the specificity and cellular resolution of miRNA expression patterns *in vivo*.

regulators [7]. Aberrant miRNA expression, which is frequently associated with cancer [1], could thus be a consequence of deregulated pri-miRNA transcription. Indeed, many genes encoding miRNAs are located at fragile chromosomal sites or chromosomal regions exhibiting altered copy numbers or genetic rearrangements in cancer [11]. The identification of common putative regulatory motifs upstream of human or *C. elegans* miRNAs [12] further suggests that certain transcription factors, which might be mutated or otherwise misexpressed in cancer, could act as master regulators of miRNA transcription. One example is MYC, which transcriptionally represses a large number of mouse and human miRNA genes [13] and induces others [14]. Epigenetic mechanisms might also contribute to silencing of certain miRNAs through histone deacetylation and DNA methylation [10].

After transcription, the nuclear pri-miRNA is cleaved endonucleolytically ('cropped'; Figure 1) by the 'microprocessor' complex consisting of the RNase Drosha and its cofactor, the double-stranded RNA (dsRNA)-binding protein DGCR8 ('DiGeorge syndrome critical region gene 8'; named Pasha in flies and worms). Cropping seems to be tightly coupled to transcription [15,16] and, for intronic miRNAs, occurs before host intron splicing [16,17]. Cropping releases the precursor miRNA (pre-miRNA), which is 60–70 nt in length and characterized by its stem–loop structure [7,8]. The pre-miRNA is exported by the nuclear export receptor exportin-5 (Ran-binding protein 21) into the cytoplasm, where Dicer excises a ~22 nt duplex RNA, from which one strand will subsequently be selected as the mature miRNA and incorporated into RISC.

Although Dicer alone is sufficient to cleave pre-miRNAs *in vitro*, it associates with various proteins in the cell, including TRBP2 (transactivation response element RNA-binding protein 2), PACT (PRKRA; interferon-inducible dsRNA-dependent protein kinase activator A) and AGO proteins (also known as EIF2C proteins) to perform pre-miRNA cleavage, mature strand selection and loading onto an AGO protein to form an active RISC [18]. At the core of RISC is a member of the AGO family, which binds the mature miRNA. In humans, there are four AGO proteins, AGO1–AGO4, among which only AGO2 has the 'slicer' activity required to cleave endonucleolytically target mRNAs that are perfectly complementary to miRNAs [19]. However, perfect complementarity between miRNAs and their targets is rare in animals, and all four AGO proteins can mediate repression of partially complementary target mRNAs through slicer-independent mechanisms that are not well understood but seem to involve translational repression, deadenylation and degradation of the target mRNAs [2].

Conceivably, transcription of a pri-miRNA could inevitably result in the expression of an active, mature miRNA, and early miRNA profiling experiments used pri-miRNA levels as a proxy for mature miRNA accumulation [20,21]. However, given the complexity of miRNA biogenesis, mature miRNA accumulation and activity could also be highly regulated. Indeed, discrepancies between the levels of the different processing intermediates and mature miRNAs [22,23] soon hinted at a regulated maturation process. Recent studies have begun to uncover various factors and mechanisms at work, suggesting that such regulation is widespread and physiologically relevant.

Regulation of pri-miRNA processing by Drosha

Although aberrant transcription can cause abnormal miRNA expression patterns, transcriptional regulation is not the only and possibly not even the most important layer of regulation. Initial evidence for this was twofold: first, in addition to deregulation of individual miRNAs, a global decrease in miRNA levels was shown to occur in diverse cancers [24]. Short of a master miRNA transcription factor that would affect transcription of many or all miRNAs, a post-transcriptional block at one or several steps of miRNA maturation seemed to be a more viable explanation for this observation. Second, although normal and tumor tissues were clearly distinguishable by their different levels of

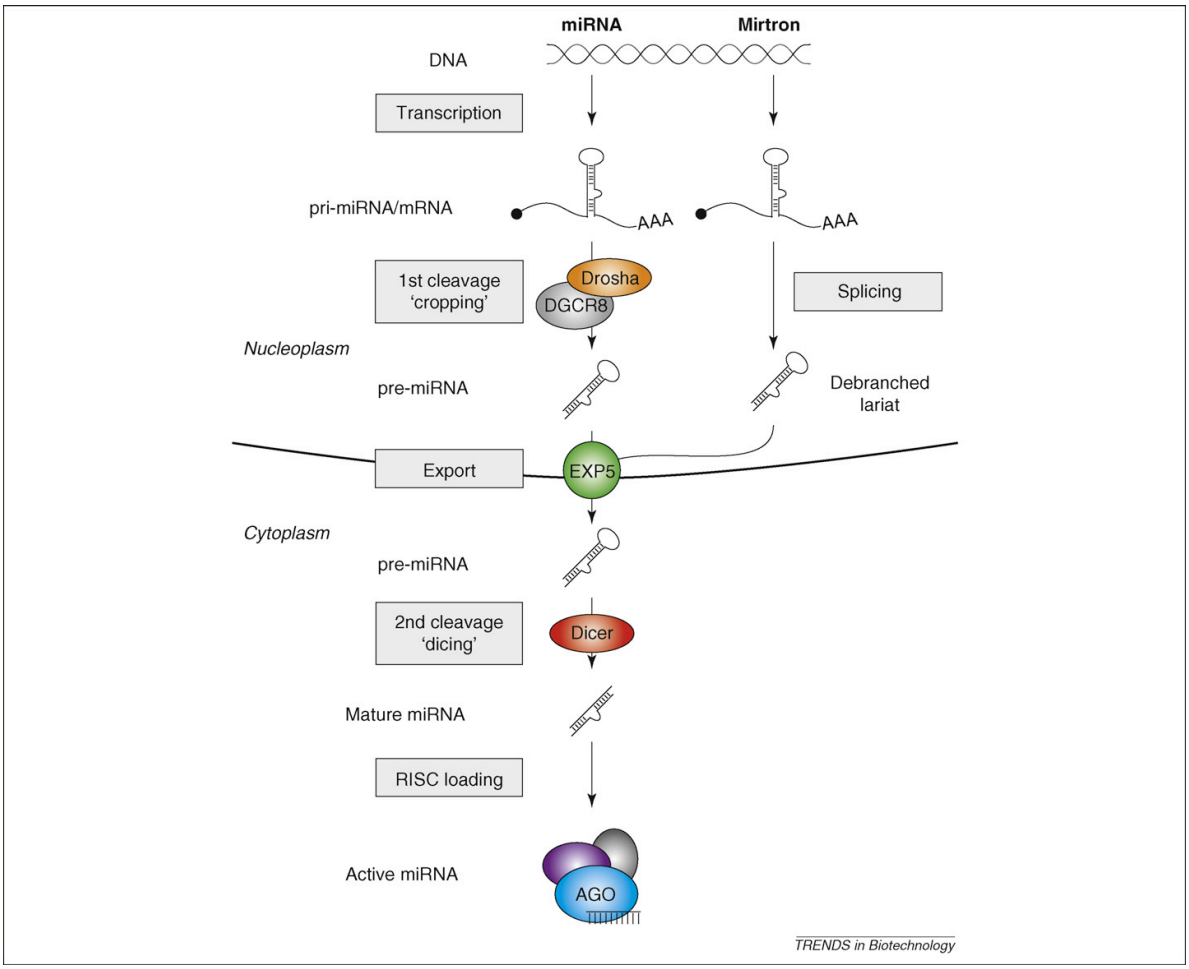

Figure 1. Schematic view of miRNA biogenesis. miRNAs are initially transcribed by RNA polymerase II as long primary transcripts (pri-miRNA) of several hundred nucleotides, either from intergenic regions or as part of protein coding genes. Pri-miRNAs are subsequently cleaved into shorter precursor miRNAs (pre-miRNA) of around 70 nucleotides by the microprocessor complex containing the RNase Drosha and its cofactor DGCR8 ('cropping'). An alternative pathway is represented by the so-called 'mirtrons' which are directly spliced out from host genes as pre-miRNA and therefore do not depend on Drosha processing. Pre-miRNA are subsequently exported, in a RanGTP (Ran-guanosine-triphosphatase bound to GTP)-dependent fashion, by exportin-5 (EXP5) to the cytoplasm, where the second maturation cleavage by the RNase Dicer takes place ('dicing'), producing 22-nt-long duplexes. By selectively loading one strand onto an argonaute (AGO) protein, the RNA-induced silencing complex (RISC) is formed and can now bind to, and repress, target mRNAs containing sites of partially complementarity to the miRNA. Each of these successive maturation steps allows for regulation in a general or miRNA-specific manner.

mature miRNA expression, pri-miRNA expression patterns indicated little difference, again pointing to differences in miRNA biogenesis activities, possibly at the level of pri-miRNA cropping by Drosha [25]. Such an effect could conceivably be a consequence of gene copy number variations or other mutagenic events that affect the expression of Drosha or its cofactors, and altered Drosha expression has indeed been observed in some tumors; for example, see Refs [26,27]. However, the fact that differentiation of embryonic stem cells also induced divergent expression levels of mature miRNAs despite comparable pri-miRNA levels provided strong evidence for a regulatory mechanism [25].

Indeed, although the Drosha–DGCR8 'microprocessor' complex is sufficient for processing of pri-miRNAs *in vitro* [28,29], cropping seems to be exquisitely modulated through accessory factors. A truncated, but functional, recombinant version of DGCR8 binds to heme, and the heme-bound, dimeric DGCR8 enhances Drosha activity towards pri-miRNA in *in vitro* processing reactions [30]. Some of this stimulation can be recapitulated when mutating the heme binding cysteine Cys352, which is conserved across animals, suggesting that heme binding stimulates Drosha–DGCR8 activity by masking a residue that normally interferes with cropping activity. It is currently unclear whether stimulation of microprocessor activity occurs also in the context of the full-length DGCR8 protein, within eukaryotic cells, and whether it is physiologically important.

In addition to its presence in the canonical Drosha–DGCR8 microprocessor complex, human Drosha has also been found in a larger complex that includes the DEAD box RNA helicases DDX5 (also known as P68) and DDX17 (also known as P72) but lacks DGCR8 [28]. Although Drosha in

the larger complex is substantially (approximately eight-fold) less active [28], this might depend on the pri-miRNA substrate. Consistent with this possibility, deletion of the genes encoding mouse DDX5 or DDX17 induces downregulation of a large, yet restricted, subset of miRNAs (94 out 266 surveyed) [31]. Among the unaffected miRNAs is miR-19a, which was also used as the substrate when comparing Drosha activity in the small versus large Drosha complex [28]. However, reduction of mature miRNA in cells lacking DDX17 and additionally depleted for DDX5 was not only limited to a subset of miRNAs but also substantially less complete than that seen upon Drosha depletion by RNA interference (RNAi), suggesting that the helicases might stimulate Drosha activity rather than being absolutely required for it.

Further support for the idea that different Drosha complexes might have different substrate preferences comes from the finding that only a subset of pri-miRNAs accumulates in *DGCR8*-hemizygous mice [32]. However, even among those miRNAs whose primary transcripts accumulated, only a single mature miRNA showed appreciable, but moderate, depletion, making the interpretation of these findings difficult. Indeed, distinct large (DDX5-, DDX17-containing) and small (DGCR8-containing) Drosha complexes have so far only been observed in human embryonic kidney 293 (HEK293) cells but not in mouse [31] or *Drosophila* cells [33]. Functional specialization of distinct Drosha complexes thus remains speculative, and confirmation will require testing of the large human Drosha complex on a wider array of substrates, including pri-mRNAs such as miR-21 whose efficient processing depends on DDX5 and DDX17 [31,34].

Understanding how DDX5 and DDX17 activate Drosha might also reveal why such stimulation is only seen for some miRNAs. Human DDX5 can unwind a synthetic hairpin structure resembling pre-*let-7a in vitro*, and this might be important for incorporation of the mature miRNA into miRISC after cleavage by Dicer [35]. Although such an unwinding function could conceivably also act on the pri-miRNA, it would seem an unlikely stimulus for the dsRNA-processing enzyme Drosha. Instead, remodeling of a hypothetical 'pri-miRNP' to displace Drosha-repressive proteins might be a more likely, but currently speculative, mode of action.

Another class of proteins that modulate pri-miRNA cropping are SMAD (small and mothers against decapentaplegic homolog) proteins [34], signal transducers best known for their function in transcriptional activation of various target genes in response to transforming growth factor β (TGF-β) signaling [36]. TGF-βs and other members of the TGF-β superfamily, such as bone morphogenetic proteins (BMPs), can bind and activate transmembrane receptor kinases, which in turn phosphorylate receptor SMADs (R-SMADs), that is, SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8. Phosphorylated R-SMADs associate with their shared 'Co-SMAD', SMAD4, to form heterodimers that accumulate in the nucleus and induce transcription of target genes. This signaling pathway functions in many developmental processes, for instance in muscle cell differentiation. Unexpectedly, recent work has identified a role of SMAD proteins in miRNA maturation that seems to be independent of their transcriptional function: treatment of human pulmonary artery smooth muscle cells with BMP4 or TGF-β1 stimulates SMAD binding to the pri-miRNA and increases Drosha activity in the extract [34]. This function is specific for miR-21 and miR-199a and requires DDX5, which can bind to R-SMADs. The mechanism of Drosha activation has not yet been identified, but increased co-immunoprecipitation of activated SMAD1 and SMAD5 with both Drosha and DDX5 suggests that SMAD proteins might help to recruit DDX5 to the microprocessor, or vice versa. However, SMAD proteins are unlikely to function as simple switches because loss of DDX5 reduces mature miR-21 levels independently of ligand [31] and both Drosha and SMAD proteins can bind to DDX5 in the absence of TGF-β, albeit less efficiently [28,34,37]. At any rate, given that previous work has identified a Drosha complex that processes pri-miRNAs inefficiently and contained DDX5 and DDX17 but not DGCR8 [28], it will be interesting to determine whether SMAD proteins and TGF-β signaling affect the DGCR8-binding status of the P68-Drosha.

Although the mechanistic details of Drosha activation by SMAD proteins thus await clarification, Davis and colleagues [34] make a strong case for its physiological relevance in promoting smooth muscle cell differentiation. This process depends on BMPs and TGF-β, and inhibition of miR-21 can partially block this effect, at least in part through causing upregulation of the miR-21 target programmed cell death protein 4 (PDCD4).

A particularly well-studied example of regulated miRNA maturation involves repression of *let-7* miRNA biogenesis by LIN28 and its paralog LIN28B. miRNAs of the *let-7* family are important regulators of stem cell self-renewal and differentiation that are absent from embryonic and other stem cells but accumulate during differentiation [38]. By contrast, LIN28 promotes pluripotency and LIN28 and LIN28B are abundantly expressed in embryonic stem cells and hepatocellular carcinoma cells, respectively. This reciprocal expression is the result of an intricate feedback loop where LIN28 or LIN28B repress *let-7* maturation, whereas mature *let-7* represses LIN28 and LIN28B accumulation [38] (Figure 2).

Unexpectedly, the ability of LIN28 and LIN28B to block accumulation of mature *let-7* involves repression of both Dicer [39] and Drosha activities [40–42], and LIN28 and LIN28B each contain two types of RNA-binding domains, a cold-shock domain (CSD) and two retroviral-type zinc-finger domains, through which they can bind pre-*let-7* (and, by inference, pri-*let-7*) [39–42]. Although it is not yet established how LIN28-binding impairs *let-7* maturation in the cell, an attractive model suggests that LIN28 competes with Drosha and Dicer for *let-7* precursor binding and prevents their access through steric hindrance. This notion is consistent with the observations that LIN28 or LIN28B are abundantly expressed in stem cells and exhibit *in vitro* binding affinities to pre-*let-7* in the high submicromolar range [40,42], and *in vitro* binding experiments have indeed confirmed that LIN28 can compete with Dicer for pre-*let-7* binding [39]. However, recent data also suggest that LIN28B might prevent pre-miRNA processing by inducing its degradation through 3′ terminal oligouridylation (see 'Note added in proof' below).

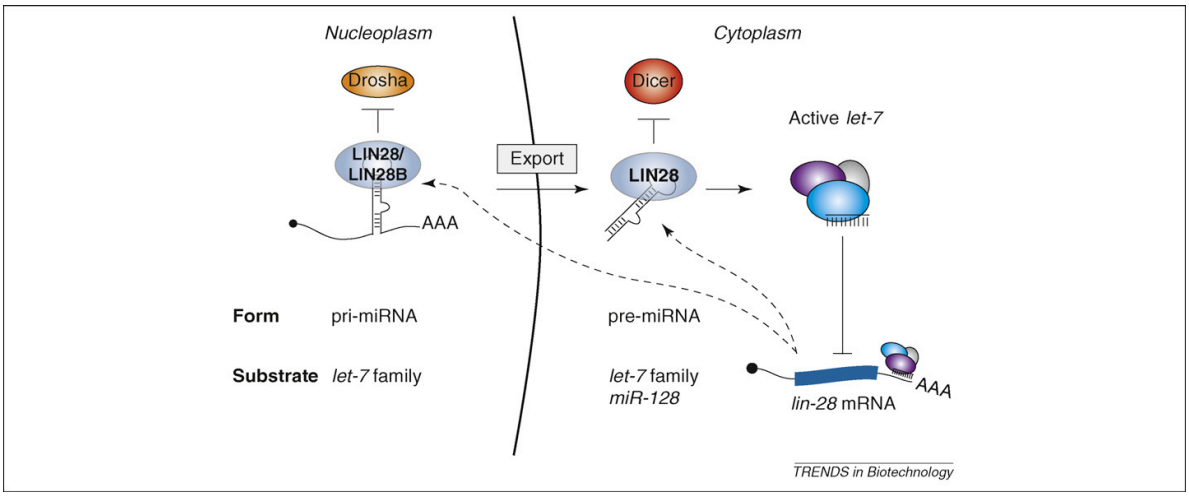

Figure 2. Regulation of miRNA processing by LIN28. LIN28 and its paralog LIN28B compete with Drosha for binding to pri-miRNAs of the *let-7* family and thereby inhibit Drosha-mediated processing. A similar competition between LIN28 or LIN28B and Dicer prevents the processing of certain pre-miRNAs in the cytoplasm. LIN28 and LIN28B also promote 3′ terminal uridylation of the pre-miRNAs, which thus become resistant to processing by Dicer and undergo degradation. The fact that the mature *let-7* miRNA targets LIN28 and LIN28B mRNAs to prevent their expression establishes a feedback mechanism.

Specificity for LIN28 binding to *let-7* miRNAs is provided by the pre-miRNA loop sequence, which contains certain nucleotides that are invariant among pre-*let-7* miRNAs, although there is some disagreement on the precise identity of the nucleotides involved [41,42]. Interestingly, the substrate specificity of LIN28 in inhibiting dicing seems wider than for cropping, as dicing of pre-miR-128, not a *let-7* family member and without obviously conserved regions in its loop sequence, is also affected [39]. Nonetheless, LIN28 does not cause a general block of Dicer activity because additional miRNAs, such as miR-125, are insensitive to increased LIN28 levels.

As discussed elsewhere [38], *let-7* accumulation is not only regulated by LIN28 and LIN28B but also itself regulates the expression of LIN28 and LIN28B through *let-7* binding sites in their 3′UTRs. Moreover, the mature *let-7* RNA can compete with pre-*let-7* for LIN28 binding *in vitro* and might thus further regulate LIN28 protein activity *in vivo* [39]. These findings suggest that an exquisite regulatory loop exists to promote a fast and stable switch from situations of low to high *let-7* levels, consistent with the reciprocal expression patterns of *let-7* and LIN28 and their opposing roles in regulating stem cell self-renewal and differentiation [38].

The heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) exhibits an even narrower substrate specificity than that of LIN28. This single-strand RNA-binding protein seems to stimulate exclusively maturation of miR-18a, although miR-18a is contained in pri-miR-17∼92, which harbors five additional miRNAs [43]. Moreover, hnRNP A1 seems to be dispensable for cropping of miR-18b, which is encoded in a distinct pri-miRNA cluster, although the pre-miRNA sequences of miR-18a and miR-18b are highly similar. Experiments on chimeric pri-miRNAs indicate that the specificity for miR-18a involves sequences outside the pre-miRNA cropping product, but how these sequences function and how hnRNP A1 stimulates pri-miR-18a cropping remains to be determined. These results demonstrate that regulation of pri-miRNA processing can extend down to the level of individual miRNAs transcribed as part of a larger cluster. Because miR-17∼92 is an oncogene [1], it will be interesting to determine in future experiments whether hnRNP A1 can modulate the tumorigenic activity of the cluster.

In the past two years, there have thus emerged several examples of modulated cropping as a means of regulating miRNA accumulation, and we can expect more in the future. For instance, processing of pri-miR-155 (B-cell integration cluster [BIC] RNA) occurs efficiently in HEK293 cells but much less efficiently in Burkitt lymphoma-derived Ramos cells [44], although this might depend on the specific experimental settings [45]. Moreover, a new class of intronic miRNAs (called ‘mirtrons’) has recently been discovered that does not require Drosha for biogenesis because splicing releases the corresponding pre-miRNA stem–loop structures (Figure 1) [46–48]. Their expression, in which the need for Drosha is bypassed, could thus be regulated in a very distinct manner from that of canonical miRNAs.

Pre-miRNA processing

After cropping of the pri-miRNA by Drosha in the nucleus, the resulting pre-miRNA is exported into the cytoplasm (Figure 1), where Dicer mediates its conversion into the mature miRNA. Dicing typically seems to function quite efficiently, as indicated by low levels of pre-miRNAs relative to mature miRNAs [49,50]. Nonetheless, early experiments already identified individual pre-miRNAs that were more abundant than their corresponding mature forms [51], although it was unknown whether this reflected regulation or simply inefficient processing of particular substrates under all conditions.

The first evidence that ‘dicing’ of specific pre-miRNAs can be regulated was obtained for mouse miR-138 [52]. Pre-miR-138 was easily detectable in many tissues, whereas the mature miR-138 derived from this precursor accumu-

lated only in mouse brain and mouse fetal liver. Processing reactions with recombinant Dicer ruled out that pre-miR-138 *per se* was refractory to processing, advocating the presence of a specific inhibitor of pre-miR-138 processing in non-neuronal tissues and cells. This activity has now been found enriched in the cytoplasm, where pre-miR-138 is also preferentially localized, ruling out that dicing is prevented by nuclear retention of pre-miR-138 (P. Leuschner and J. Martinez, personal communication). The molecular identity of the repressor has yet to be established.

As discussed in the previous chapter, dicing of *let-7* family pre-miRNAs is also regulated, and the inhibitor has been identified as LIN28 (Ref. [39]) – the same protein that also inhibits cropping of pri-*let-7* (Figure 2). Indeed, regulation of dicing might be quite common: in a large-scale profiling effort, expression of precursor and mature forms of 201 miRNAs correlated poorly across various human tissues and cell lines [49]. For instance, mature miR-128a accumulates almost exclusively in brain and skeletal muscle but not in spleen or B cells. However, pre- and pri-miR-128a levels (which were not distinguished in this PCR-based experiment) were considerably higher in spleen and B cells than in skeletal muscle, albeit lower than in the brain. *In situ* hybridization in cancer cell lines that did not accumulate mature miR-128a, miR-105 or miR-31 detected exclusively nuclear and/or nucleolar signals, with probes detecting both pri- and pre-miRNAs [49]. In a cell line that accumulated mature miR-31, such a probe revealed additionally some cytoplasmic, but no nucleolar, signal. The authors suggest that the nuclear and/or nucleolar signal reflects pre-miRNA retention, preventing access by cytoplasmic Dicer. How the nuclear export of this pre-miRNA is regulated is not known. Moreover, because northern blot analysis demonstrated that long transcripts, presumably pri-miRNAs, were abundant in cells lacking the mature miRNA, these species might contribute a significant nuclear hybridization signal.

A more general regulation of Dicer activity than in the examples discussed above is conceivable. For instance, Dicer can be proteolytically activated [53], possibly by relieving the autoinhibitory function exerted by its helicase domain [53,54]. Although this activation seemed to be more substantial for dsRNA substrates than for pre-miRNAs [54], such a mechanism might affect miRNA accumulation in the brain [55]. If Dicer activity is limiting, or close to limiting, for pre-miRNA processing, regulation of Dicer expression might also permit modulation of pre-miRNA processing, an intriguing possibility given that Dicer expression or gene copy number is altered in diverse cancers [56]. 'Decoy substrates' that compete with Dicer substrates for access to this nuclease but that, unlike true substrates, are not cleaved might further regulate Dicer activity. For instance, overexpression of the RNA *rncs-1* (*RNA noncoding, starvation upregulated*) in *C. elegans* reduces Dicer-dependent endogenous small interfering RNA (endo-siRNA) accumulation [57]. When added to embryo extract, this 800-nt-long dsRNA with structured ends reduces siRNA generation from a long dsRNA substrate without itself being cleaved. However, because processing of pre-*lin-4* miRNA was not affected, it remains to be established whether such mechanisms also operate to regulate pre-miRNA dicing.

Finally, efficient pre-miRNA processing involves additional proteins, such as AGO proteins, TRBP2 and PACT [18,58–60], so regulation of these proteins might further modulate Dicer activity. Interestingly, stability of human AGO2 has recently been shown to be affected by proline hydroxylation through the type I collagen prolyl-4-hydroxylase (C-P4H(I)) [61]. However, although C-P4H(I) depletion reduced RISC activity, as determined by decreased cleavage of a perfectly complementary *let-7* target reporter, it is not known whether AGO2 destabilization coincided with a depletion of mature miRNAs. More importantly, because repression of a partially complementary miR-21 target reporter remained unaffected by both AGO2 and C-P4H(I) depletion, it remains to be established that this modification significantly modulates repression of authentic miRISC targets. Nonetheless, this new finding and the studies discussed above illustrate that there is great potential for regulation of miRNA maturation at the level of 'dicing', and we are just beginning to understand some of the mechanisms at work.

miRNA turnover

Surprisingly, several of the events affecting processing of intermediates along the miRNA biogenesis pathway do not alter accumulation of the respective processing substrates. For instance, LIN28 blocks processing, and thus consumption, of pri-*let-7* in stem cells, yet pri-*let-7* levels are equal in cells with and without LIN28 [25]. Similarly, increased pri-miR-21 processing upon stimulation by TFG-β or BMP4 does not alter pri-miR-21 levels [34] and nor does loss of DDX5 and DDX17 [31]. One possible explanation is that, in each of these cases, sufficient transcriptional stimulation might balance out increased consumption and, conversely, decreased consumption might be paralleled by decreased transcription. Alternatively, turnover of unprocessed, 'naked' transcripts might be very fast so that binding by the respective processing factors is required to sufficiently stabilize the substrate. The experimental investigation of pri-miRNA transcription rates could help to distinguish between these – not mutually exclusive – possibilities. Whether such putative turnover of miRNA processing intermediates is a regulated or constitutive process remains an open question for the time being.

In addition to miRNA processing intermediates, degradation of mature miRNAs might also affect the net accumulation of mature miRNAs. Possible examples include stabilization of miR-122a by the DNA and RNA-binding translin (TSN) protein [62] or general destabilization of mature miRNA levels by the Trim-NHL (tripartite motif, NCL-1, HT2A and LIN-41 domain) protein and Ago1 binding partner Mei-P26 (meiotic gene recovered in a P-element screen) in *Drosophila* stem cells [63]. Moreover, cell-cycle status affects the stability of miR-29b, which is rapidly degraded in proliferating cells but stabilized in mitotically arrested cells [64]. However, mechanisms have not been established for any of these events, and there is only limited evidence that these proteins do indeed affect miRNA turnover as opposed to a late biogenesis step.

Box 2. Regulation of microRNA maturation and activity by RNA editing

Adenosine deaminases acting on RNA (ADARs) can convert adenosine to inosine (A-to-I editing) in various double-stranded RNA substrates, and several studies have demonstrated that both pri-miRNAs and pre-miRNAs can be substrates of ADARs [84,85] (Figure 3). For instance, selective editing of human pri-miR-142 blocks its processing by Drosha [86]. The unprocessed pri-miR-142 is rapidly degraded *in vitro* by Tudor staphylococcal nuclease homolog (Tudor-SN). As discussed elsewhere [87], this suggests that editing of specific pri-miRNA might be common but fails to be detected owing to efficient degradation, an idea that is also supported by a recent study identifying 47 pri-miRNAs that are edited without usually giving rise to edited mature miRNAs [85]. Editing also prevents the dicing of both mouse and human miR-151 [88], which therefore accumulate as pre-miRNAs. How editing blocks processing and to what extent this mechanism is used and possibly regulated remains to be elucidated.

In addition to affecting miRNA maturation, editing can also affect the specificity of the edited miRNAs if the edited site is included in the mature miRNA, because inosine base-pairs with cytosine instead of uracil. A survey of 99 miRNAs in human tissues revealed that six of these were edited in at least one tissue, and a substantial number of these editing events were predicted to modify target recognitions [89]. Experimental confirmation of this hypothesis was provided for miR-376, which is edited by both ADAR1 and ADAR2, at different positions of the precursor [90]. ADAR2-mediated editing of a nucleotide corresponding to position +4 in the mature miR-376-5p in particular creates a miRNA that, unlike its unmodified counterpart, represses phosphoribosyl pyrophosphate synthetase 1 (PRPS1) expression and its resulting enzymatic activity *in vivo*.

Intriguingly, a different type of editing enzyme, the cytidine deaminase APOBEC3G (ABC3G), was found to counteract the activity of several miRNAs [91]. However, this function does not depend on the deaminase activity of ABC3G, leaving it unclear how miRNA inactivation is achieved.

Although a recent deep-sequencing effort provided little evidence for widespread A-to-I editing of miRNAs [82], editing can play a crucial part in modifying the activity of a subset of miRNAs and thus needs to be considered when evaluating the potential relevance of miRNA-mediated gene regulation *in vivo*.

Although there is thus little known about any mechanisms of either constitutive or regulated degradation of mature miRNAs in animals, recent work in plants has identified the small RNA degrading nuclease SDN1, a homolog of the yeast 3′-to-5′ exonucleases Rex1p (RNA exonuclease 1) to Rex4p, as a nuclease that can degrade 'naked' small RNAs such as miRNAs *in vitro* [65]. Combined knockdown of three nucleases of the SDN1 family in *Arabidopsis* causes a two- to threefold increase in the levels of several mature miRNAs, suggesting that these miRNAs are also substrates of this family of RNases *in vivo*. However, it is currently not known whether the activity of these RNases is regulated and whether enzymes of this family affect miRNA stability in animals.

Concluding remarks

Although regulation of miRNA transcription is clearly important for setting miRNA expression patterns, the work that we have discussed here demonstrates that additional, post-transcriptional mechanisms further define miRNA activity, at least for a subset of miRNAs and tissues. Other mechanisms, such as RNA editing (Box 2 and Figure 3), can affect miRNA maturation and function, as will mechanisms that have not yet been discovered. However, one conclusion that we can already draw is that the levels of miRNA pathway intermediates will frequently not be good proxies for the expression patterns of mature miRNAs, which therefore need to be examined directly (Box 1).

In addition, if we want to understand the roles of miRNAs in specific cells or organisms and harness the regulatory power of miRNAs at a diagnostic or even a therapeutic level (Box 3), we need to consider additional factors that determine miRNA activity. These include 'static' factors such as single nucleotide polymorphisms and mutations, either in the miRNA or its targets, that might generate or abolish miRNA binding sites or affect their effectiveness (e.g. [66,67]). More importantly, 'dynamic' factors modulate miRNA activity without altering mature miRNA concentrations, for instance in

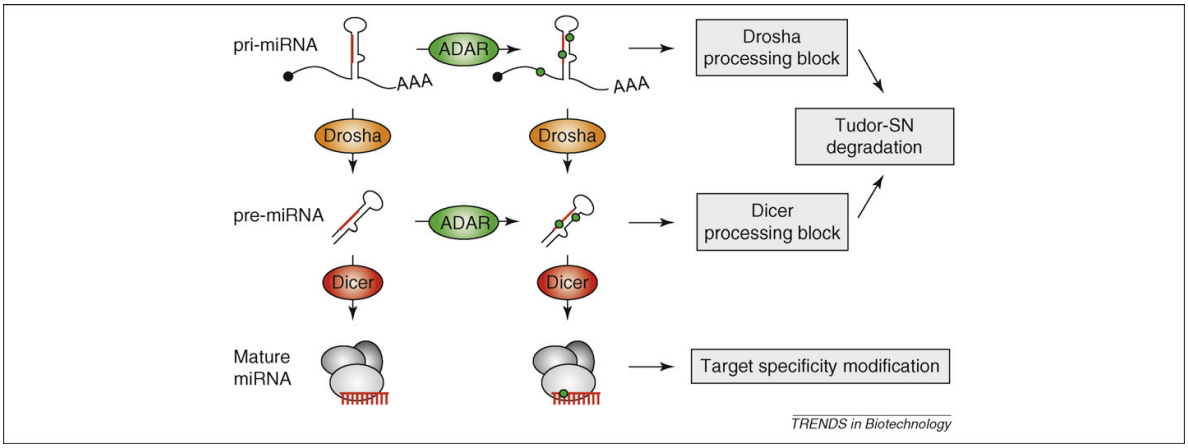

Figure 3. Modulation of miRNA maturation and activity by RNA editing. Both pri- and pre-miRNA can be substrates of adenosine deaminases acting on RNA (ADARs), which convert adenosine to inosine. Editing (green dots) of pri-miRNAs and pre-miRNAs can prevent efficient processing by Drosha or Dicer and/or direct the edited molecules to Tudor staphylococcal nuclease homolog (Tudor-SN) for degradation. Alternatively, editing of pri- or pre-miRNAs can also lead to the accumulation of modified mature miRNAs (red lines) that can potentially recognize a different set of targets compared to the unedited miRNAs.

Box 3. MicroRNA-based therapeutic approaches

Recent findings that antisense oligonucleotides can specifically block a given miRNA *in vitro* and *in vivo* have triggered efforts to explore miRNAs as a potential new class of therapeutics [92]. A recent study demonstrated that intravenous injection of a short locked nucleic-acid-modified oligonucleotide ('LNA-anti-mir') could block miR-122 activity in African green monkey, accompanied by a dose-dependent and long-lasting lowering of plasma cholesterol. Importantly, there was no evidence for LNA-anti-miR-associated toxicity or histopathological changes in the studied animals [93], and a phase I safety study in humans has been initiated.

Conversely, restoring miRNA function by the exogenous delivery of a synthetic miRNA mimic might have therapeutic benefit, in particular in certain cancers where tumor-suppressor miRNAs are under-represented. Two recent studies demonstrated that viral delivery of *let-7* reduced tumor formation in mouse models of lung cancer [94,95]. However, this work also suggested that tumors might gain resistance to *let-7 g* expression [95].

Many miRNAs are believed to act in a highly tissue- or even cell-type-specific manner, where they contribute to the establishment and maintenance of cellular identity [7]. Furthermore, a significant number of miRNAs are coexpressed as transcription units and might act in a combinatorial manner. Thus, targeting a subset of miRNAs might be necessary to significantly modulate one given mRNA target, and potential adverse effects caused by potential off-targeting events remain to be carefully assessed. By contrast, a miRNA inhibitor that modulates a whole disease-relevant pathway by reducing several functionally related genes at once could in theory be more effective than the more conventional agents tailored to target one single protein. Finally, as for related antisense and siRNA-based approaches, developers of miRNA therapeutics face the crucial obstacle of ensuring both the stability of the inhibitors in the body and, most importantly, delivery to, and uptake by, the organ or cells of interest [96]. Although initial experiments in these directions look encouraging [97], much more work needs to be done to demonstrate that the concept of tuning miRNA levels is clinically viable.

response to cellular growth phase or stress. Examples are the use of alternative forms of 3′UTRs, with different complements of miRNA target sites, by mRNAs [68,69], expression of 3′UTR binding proteins that affect accessibility of miRNAs even to those target sites that are present in a given 3′UTR [70,71], 'decoy' RNAs that sequester miRNAs away from their target [72] or, most dramatically, a switch from target repression to target activation by miRISC itself [73]. Some of these mechanisms can even function post-repression, that is, they can derepress mRNAs that have been translationally silenced by specific miRNAs [70], and such induced reversal of miRNA-mediated gene silencing might be particularly important for activation-dependent, localized translation at neuronal synapses [74]. Additional mechanisms such as 'target protection' [75] – that is, blocking of an miRNA target site through binding of an oligonucleotide that overlaps the site – are only speculative at present but work already as experimental tools, making it tempting to propose that cells might employ similar strategies as well.

Unraveling these complex networks of dynamic regulatory interactions will remain a major challenge for some time to come and will require the use of both defined cell-based assays and physiological systems, such as whole-animal models. At the same time, the exquisite post-transcriptional regulation of individual miRNAs might provide unexpected opportunities for targeted manipulation of miRNA expression as a promising complement to antisense or RNA-based therapeutic approaches (Box 3). Indeed, a first step towards exploiting this opportunity has just been reported: enoxacin, a low-molecular-mass antibacterial agent, promotes accumulation of a small subset of mature miRNAs, apparently through stimulating pre-miRNA processing by TRBP2–Dicer and subsequent RISC loading [76].

Note added in proof

LIN28B has recently also been observed to block dicing of pre-*let-7* in hepatocellular carcinoma cell lines [77]. Direct competition between Dicer and LIN28 or LIN28B seemed to contribute relatively little to repression of processing; instead, binding by LIN28 or LIN28B promoted 3′ terminal oligouridylation of pre-*let-7* by an unidentified terminal uridyl transferase. The oligouridylated pre-*let-7* was resistant to Dicer processing *in vitro* and underwent degradation, providing a novel mechanism for inhibition of *let-7* maturation.

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2.2.4. microRNA modes-of-action

The nature of the mechanism by which animal miRNAs repress their targets is certainly the most debated aspect of miRNA biology. A plethora of models, sometimes mutually exclusive, has been proposed over the last years and have been the subject of an intense discussion in various reviews (Jackson and Standart 2007; Nilsen 2007; Pillai et al. 2007; Standart and Jackson 2007; Eulalio et al. 2008a; Filipowicz et al. 2008; Richter 2008; Wu and Belasco 2008; Carthew and Sontheimer 2009; Chekulaeva and Filipowicz 2009). Moreover, some studies suggest that, under specific conditions, miRNAs act as translational activators rather than repressors (Vasudevan et al. 2007; Henke et al. 2008; Orom et al. 2008). Admittedly, miRNAs might function via different mechanisms depending on the organism, cell type, miRNA, miRNA target, or conditions investigated. However, even studies reporting very similar experiments sometimes reached opposite conclusions (e.g. (Petersen et al. 2006) and (Humphreys et al. 2005)). Therefore, one can hope that a deeper understanding of the mechanisms at play might help, at least partially, to unify the large body of apparently conflicting data, which exists today.

In plants, miRNAs, defined as such based on their biogenesis, were thought to act essentially like siRNAs, that is to base-pair perfectly or almost perfectly to target transcripts, and to induce endonucleolytic cleavage (Llave et al. 2002; Rhoades et al. 2002). This simple picture was recently challenged by Brodersen and colleagues, who uncovered mutations in *Arabidopsis,* which induce accumulation of miRNA targets at the protein level without changes at the mRNA level, and proposed that plant miRNAs function partially also as translational repressor (Brodersen et al. 2008). However, this study and previous ones, which documented similar examples (Aukerman and Sakai 2003; Chen 2004; Gandikota et al. 2007), did not investigate directly the translational status of miRNA targets. Therefore, alternative mechanisms, such as nascent polypeptide degradation, cannot be excluded yet.

Coming back to animal miRNAs, initial studies aimed at understanding their mode-of-action were performed in *C. elegans*, focusing on the first identified miRNA, *lin-4* and two validated targets: *lin-14* and *lin-28* (Olsen and Ambros 1999; Seggerson et al. 2002). This studies established that, if LIN-14 and LIN-28 protein levels are downregulated by *lin-4*, *lin-14* and *lin-28* mRNAs are not significantly degraded and their polyadenylation status and

polyribosome association not substantially modified. Based on these observations, it was initially assumed that miRNAs function solely by inhibiting target translation at a step downstream on initiation without affecting the amount or structure of target mRNAs themselves.

Surprisingly, subsequent studies showed that miRNA targets often undergo a significant amount of degradation, most likely triggered by deadenylation. These include studies conducted in animal models (Bagga et al. 2005; Krutzfeldt et al. 2005; Giraldez et al. 2006; Mishima et al. 2006), cell lines (Lim et al. 2005; Behm-Ansmant et al. 2006; Rehwinkel et al. 2006; Schmitter et al. 2006; Wu et al. 2006; Eulalio et al. 2007b; Eulalio et al. 2009b) or cell-free system (Wakiyama et al. 2007). Based on these results, it is today generally accepted that miRNAs can, to a certain extent, induce target degradation. miRISC components, such as Ago proteins and by extension miRNAs, together with miRNA targets were shown to colocalize with processing-bodies (P-bodies), which are cellular foci enriched for the RNA degradation machinery (reviewed in (Parker and Sheth 2007)). Although P-bodies integrity is not required for miRNA mediated repression (Eulalio et al. 2007a), it was shown in *Drosophila* cells that the P-body components CAF1:CCR4:NOT1 (deadenylase complex), DCP1:DCP2 (decapping complex), and Xrn1 (exonuclease) mediate miRNA targets degradation (Behm-Ansmant et al. 2006; Eulalio et al. 2007b; Eulalio et al. 2009b).

It is also generally acknowledged that miRNA can repress the translation of their targets in parallel with, or prior to, mRNA degradation. However no agreement could be reached so far about the step of translation that is inhibited (Chekulaeva and Filipowicz 2009). Early experiments support a post-initiation mechanism (Olsen and Ambros 1999; Seggerson et al. 2002). This is essentially based on the observation that target mRNA distributions in sucrose density polysome gradients is unchanged whether or not the cognate miRNA is expressed, indicating that the repressed and non-repressed mRNAs are in average loaded with the same number of ribosomes. It was also shown that some miRNAs and miRISC components co-sediment with polysomes in human neuronal cells, suggesting that repressed targets are still loaded with ribosomes (Nelson et al. 2004). A following study by Pillai and colleagues fired up the controversy (Pillai et al. 2005). The authors showed that in HeLa cells *let-7* binding to reporters mRNA shifts them to lighter fractions of density polysome gradient, indicating that

less ribosomes are present on the repressed mRNAs, a hallmark of reduced translation initiation rates. Moreover, this study and another one agreed on the observation that reporters driven by internal ribosome entry sites (IRESs), i.e. reporters, which do not rely on a cap structure for their translation, are refractory to miRNA-mediated repression, suggesting that miRNAs prevent interaction between the mRNA cap and translation initiation factors (Humphreys et al. 2005; Pillai et al. 2005). However, the same two studies disagreed about whether a poly(A) tail is required for repression.

Adding to the confusion, a set of subsequent studies using human cell lines argued in favor of a post-initiation phenomenon, although suggesting different models (Maroney et al. 2006; Nottrott et al. 2006; Petersen et al. 2006; Lytle et al. 2007). In these studies, arguments for a post-initiation mechanism are essentially based on observations that repressed mRNAs together with miRNAs sediment in deep (polysomal) fractions of polysome gradients and that reporters driven by certain types of IRES can still be repressed by miRNAs. Data from Maroney *et al*. suggest that repressed targets are actively translated and implicitly point toward a mechanism affecting the nascent polypeptide (Maroney et al. 2006). Such a model is also explicitly supported by Nottrott and colleagues, however direct experimental evidences are lacking (Nottrott et al. 2006). Peterson and co-workers proposed that translational repression is achieved by premature ribosome drop-off (Petersen et al. 2006). However, they do not see repressed targets shifting to lighter polysome fractions, something that, based on this model, would be expected as pointed out by Chekulaeva and Filipowicz (Chekulaeva and Filipowicz 2009). Simultaneously, other studies substantiated the initiation model, using globally the same experimental strategies as the studies mentioned above but finding opposite results (Bhattacharyya et al. 2006; Huang et al. 2007). Importantly, Bhattacharyya and colleagues reported the first observation of an endogenous target, CAT-1, being repressed at the initiation level in human cells (Bhattacharyya et al. 2006).

Several laboratories recapitulated, at least to some extent, miRNA repression in cell-free systems (Wang et al. 2006; Mathonnet et al. 2007; Thermann and Hentze 2007; Wakiyama et al. 2007). All these studies point to an initiation mechanism, based on the fact that reporters containing either a non-functional cap or an IRES cannot be repressed by miRNAs. The

initiation model was also further supported by two additional studies, while each implying a different mode-of-action (Chendrimada et al. 2007; Kiriakidou et al. 2007).

By immunoprecipitation, Chendrimada *et al*. identified an interaction between miRISC and the eukaryotic translation initiation factor 6 (eIF6), a factor involved in preventing the premature association between the 60S and 40S ribosomal subunits. Depletion of eIF6 prevented miRNA-mediated repression of reporter mRNAs in human cells and of the *lin-4* targets *lin-14* and *lin-28* in *C. elegans,* suggesting that miRNAs inhibit translation initiation by preventing the 60S ribosomal subunit joining (Chendrimada et al. 2007). This model was supported by experiments in reticulocyte lysate which showed that miRNA targets are enriched for 40S, but not 60S, ribosome components (Wang et al. 2008). However, as noted earlier (Filipowicz et al. 2008), the fact that eIF6 is involved in ribosome biogenesis renders these data difficult to interpret, as translation could be generally affected by eIF6 depletion. Chendrimada *et al*. argue that eIF6 depletion does not affect global translation, as determined by polysome profiling, however the quality of these experiments is questionable: polysomes are almost completely absent in every conditions tested, making it difficult to assess any change in polysome magnitude. Finally, if eIF6 really plays a role in miRNA-mediated repression, it does not seem to be conserved in *D. melanogaster*, where no effect on miRNA target reporters could be observed upon eIF6 depletion (Eulalio et al. 2008b).

Kiriakidou and colleagues uncovered two residues highly conserved among Ago proteins, which were proposed to allow Ago to bind the mRNA cap structure. It was therefore suggested that Ago proteins directly compete with eIF4E, the cap-binding translation initiation factor, to inhibit translation initiation on miRNA targets (Kiriakidou et al. 2007). However, a recent bioinformatic study suggests that the conserved residues cannot fold in a structure able to bind the cap (Kinch and Grishin 2009). Alternatively, Eulalio *et al*. showed that these residues are important for the interaction between Ago1 and GW182, a miRISC component, in *Drosophila* cells. This result might explain why these residues are conserved and suggests that Ago-GW182 interaction is important for miRNA function (Eulalio et al. 2008b).

GW182 (a 182 kiloDaltons protein enriched in glycine and tryptophan repeats) was identified in 2002 using sera from a patient suffering from motor and sensory polyneuropathy and found to localize in discrete cytoplasmic foci named GW-bodies (Eystathioy et al. 2002).

Subsequent studies showed that GW-bodies correspond to P-bodies, indicating that GW182 might be involved in the control of mRNA stability. In parallel, co-localization and co-immunoprecipitation experiments in human cells showed that Ago proteins localize to P-bodies and interact with components of the mRNA degradation pathway and that miRNA targets are localized to P-bodies in a miRNA-dependent fashion (Liu et al. 2005b; Sen and Blau 2005). This suggested a link between GW182 and the miRNA pathway, which was confirmed by the observation that human and *Drosophila* GW182 physically interact with Ago proteins and that depletion of GW182 prevent efficient repression of miRNA target reporters (Liu et al. 2005a; Meister et al. 2005; Rehwinkel et al. 2005; Behm-Ansmant et al. 2006; Eulalio et al. 2008b). Three GW182 paralogs have been identified in vertebrates (TNRC6A/HW182, TNRC6B, TNRC6C) and one in insects (GW182 or Gwaky). *C*. elegans has also been found to encode two distant homologs of this protein family: *ain-1* and *ain-2* (*alg-1* interacting protein) (Ding et al. 2005; Ding and Han 2007; Zhang et al. 2007). Similarly to other GW182 proteins, AIN-1 and AIN-2 interact with ALG-1 and ALG-2, the *C. elegans* miRNA specific Ago proteins, and are essential for miRNA-mediated repression (Ding et al. 2005; Zhang et al. 2007). Recent efforts have been made to understand the architecture of GW182 proteins in flies and humans (Baillat and Shiekhattar 2009; Chekulaeva et al. 2009; Eulalio et al. 2009a; Eulalio et al. 2009c; Lazzaretti et al. 2009; Lian et al. 2009; Takimoto et al. 2009; Zipprich et al. 2009). These studies identified several domains required for Ago interaction and for miRNA target silencing (reviewed in (Eulalio et al. 2009d)), but did not address the mechanistic aspect of silencing itself. Therefore, although it is now manifest that GW182 proteins play a crucial role for miRNA-mediated silencing, it is unclear whether they are involved in an initiation or post-initiation inhibition mechanism. It is also surprising that AIN-1 and AIN-2 function, at least superficially, similarly to human and flies GW182 proteins, since they are only distantly related to these and lack for example the silencing domains identified in the aforementioned studies (Eulalio et al. 2009d). This suggests that either *C. elegans* AIN-1 and AIN-2 might function in a different way than other GW182 proteins or that a feature common to all of them as not yet been identified.

In summary, among the very large body of literature related to miRNA mode-of-action common themes, as well as discrepancies are apparent. Essentially, miRNA targets can be either repressed translationally or degraded or undergo a combination of both, however it is not known

if degradation requires prior translational repression. Moreover, there is no consensus about how translation of miRNA targets is impaired and, although GW182 proteins are recognized to be central to this and the degradation processes, there is little to no information about their precise molecular functions. An interesting postulate is that core miRISCs function as platforms, which could recruit different sets of effector proteins in a time-, tissue-, or environment-dependent manner, allowing miRNAs to function differently in distinct contexts (discussed in (Hammell 2008)). Such a scenario could explain that different experimental settings exhibit different type of miRNA-mediated repression, or even activation, mechanisms. In nematodes, food deprivation was shown for example to prevent *lin-4* dependent *lin-14* mRNA degradation, but not protein downregulation (Holtz and Pasquinelli 2009). Additionally, modulation activities could also be brought about by factors recruited to the 3'UTR of specific targets, such as, for instance, the RNA-binding protein Dnd1, which can mask certain miR-430 binding sites in zebrafish germ cells (Mishima et al. 2006; Kedde et al. 2007), or HuR, which can relieve CAT-1 repression by miR-122 in liver cells under stress conditions (Bhattacharyya et al. 2006). Consequently, a better understanding of potential miRISC modulators might help to reconcile apparently conflicting data.

Finally, it has to be noted that most of the studies discussed here have been conducted using artificial reporter systems and also sometimes artificial miRNAs, leaving it unclear how faithfully these systems recapitulate the endogenous miRNA-mediated regulation process. In my opinion, observations made with non natural molecules should be inferred only very cautiously to the endogenous situation. Cellular processes are robust machineries which can accommodate varying situations (Stelling et al. 2004). It is not granted that such machineries will automatically function canonically when confronted with artificial substrates. Therefore, experiments employing non-endogenous substrates can only inform us about the functional possibilities of a cellular machinery but not directly about its endogenous mode-of-action. For example, the fact that an artificial reporter mRNA containing a histone stem loop instead of a poly(A) tail can be repressed by miRNAs (Wu et al. 2006) cannot not be directly used to demonstrate that poly(A) tails of endogenous targets do not play a role in miRNA-mediated repression. Unfortunately, this distinction is very often not made when discussing such results. This concern is also further underlined by the observations that the mode of reporter transfection or the type of viral promoter used to express reporters have been shown to influence

the apparent mode of miRNA-mediated repression (Lytle et al. 2007; Kong et al. 2008) and calls for studies focusing on the physiologically relevant aspects of miRNA-mediated repression.

2.2.5. Biological functions of microRNAs

Regardless of how exactly miRNAs silence their target genes, this type of regulation has been shown to be relevant to an overwhelming large number of cellular and physiological processes in health and disease. These include developmental timing, stem cell maintenance, cellular proliferation and differentiation, apoptosis, viral infection, immune response, hematopoiesis, and angiogenesis, to name just a few (reviewed in (Bushati and Cohen 2007)). Importantly certain microRNAs have also been shown to function as tumor-suppressor or oncogene genes (reviewed in (Esquela-Kerscher and Slack 2006)). It is maybe not so surprising that the breadth of processes regulated by miRNAs is so large, considering that an average miRNA has been predicted to regulate more than hundred targets and that 20% to 30% of animal 3'UTRs contain at least one conserved miRNA predicted binding site (Krek et al. 2005; Lewis et al. 2005). These predictions are difficult to establish, due to the imperfect nature of miRNA - miRNA target binding in animals. Nevertheless, they have seen some experimental support from recent large-scale proteomics studies, which indicated that miRNAs typically repress hundreds of targets, albeit in average to a moderate extent (Baek et al. 2008; Selbach et al. 2008).

In general, miRNA interaction with their targets can be characterized according to how much a given target is repressed, how much the target repression physiologically matters, and how many targets a given miRNA represses to achieve its function. The consideration of these characteristics allows to classify miRNA activities as either "genetic switch" or "fine-tuning" (Flynt and Lai 2008). Genetic switches generally concern cases one or a few targets. This is for example the *C. elegans* L1 to L2 developmental switch controlled by *lin-4* repression of *lin-14* (see section 2.2.1.). *lin-4* expression allows worm development to proceed from the L1 to the L2 stage by almost completely abolishing *lin-14* expression. Another example of a clear developmental switch in nematodes comes from *let-7*. This miRNA, which regulates multiple targets, including various transcription factors (Grosshans et al. 2005), controls the L4 to adult transition by repressing the NHL protein LIN-41 (Reinhart et al. 2000). *let-7* mutations, depending on their penetrance, lead to various phenotypic defects ranging from cuticular defects to death by vulva bursting (Reinhart et al. 2000). Interestingly, individual depletion of *let-7* targets is often sufficient to rescue *let-7* mutant animals (Abrahante et al. 2003; Grosshans et al.

2005; Lall et al. 2006), suggesting that a complex network of genetic interactions might exist between the targets regulated by a given miRNA.

In instances of fine-tuning regulation, the role of miRNAs is to set a precise level of target activity, therefore, in this case and contrary to the *lin-4 - lin-14* paradigm, targets are often co-expressed together with their cognate miRNAs. Importantly, fine-tuning regulation is not synonymous with trivial regulation, as exemplified by the *D. melanogaster miR-8* regulation of *atrophin* (Karres et al. 2007). *miR-8* mutant phenotypes - elevated apoptosis in the brain and behavioral defects - are caused by upregulated levels of Atrophin and can be rescued by deleting one copy of the *atrophin* gene. Interestingly, knocking-down *atrophin* specifically in *miR-8* expressing cells below the level achieved by *miR-8* repression also results in phenotypic defects. This shows that *miR-8* functions by achieving a precise, physiologically optimal level of Atrophin. Another example comes from the study of B-cell differentiation. *miR-150* regulates B-cell lineage development essentially by tuning precisely, among several predicted targets, the level of the c-Myb transcription factor (Xiao et al. 2007; Zhou et al. 2007). Here as well, too low and to high c-Myb levels are equally detrimental.

Some miRNA functions have also been shown to depend on the regulation of a large set of targets. An impressive example comes from the ectopic expression of tissue specific miRNAs in HeLa cells (Lim et al. 2005). Cells transfected with the brain specific *miR-124* or the muscle specific *miR-1* specifically shift their expression profile toward that of brain or muscle cells, respectively, as determined by level modulation of about hundred transcript in each cases. This and additional studies (Farh et al. 2005; Sood et al. 2006) show that miRNAs can enforce cellular identities by modulating a large number of targets, which are often co-expressed with tissue specific miRNAs, but kept to a low level. In an other study, it appears that certain *Drosophila* miRNAs and their cognate targets are reciprocally expressed in adjacent but not overlapping tissues (Stark et al. 2005). This suggests that miRNAs, by targeting genes that are already transcriptionally inactive, might also function as a backup system repressing transcriptional leaks. Such a model might explain why a large-scale study of individual miRNA mutants in *C. elegans* reported most of them to be superficially wild-type (Miska et al. 2007). Alternatively, this could indicate that a large degree of redundancy between certain miRNAs exist. Indeed, a large number of *C. elegans* and *D. melanogaster* miRNAs have homologues

predicted to regulate identical sets of targets (Ibanez-Ventoso et al. 2008). This could also suggest that, besides a few cases of life-or-death regulation, most miRNAs regulate subtle or environment-dependent phenotypes.

Altogether, miRNA-mediated repression, by being able to quickly block the expression of mRNAs, which might have otherwise long half-lives, seems particularly well adapted to situations requiring a rapid and clear change in gene expression. miRNAs are also well suited for local regulation of translation, like in neurons for instance (Ashraf et al. 2006). Additionally the fact that, in certain cases, miRNA-mediated repression is reversible (Bhattacharyya et al. 2006), suggests that miRNAs can be particularly useful not only for a fast repression but also reactivation of their targets. Moreover, miRNAs probably represent a rather evolutionary plastic mode of gene regulation as the modification of only a few nucleotides in a transcript is sufficient to introduce or delete miRNA binding sites. This idea is supported by the observation that, although animal miRNAs are in general well conserved, only a few miRNA - miRNA target interactions are evolutionary preserved (Chen and Rajewsky 2006). These cumulative advantages of miRNAs might explain why they seem to play a role in virtually every biological process investigated so far.

2.3. Aim of this work

Considering the existing controversy about the mode-of-action of miRNAs (see section 2.2.4.) and the relative lack of *in vivo* studies on this topic, the aim of this work was to understand in more details the mechanistic aspects of miRNA-mediated repression in physiologically relevant context. This study was performed using *C. elegans* and focusing mostly on *let-7*, as a model miRNA (reviewed in (Bussing et al. 2008)). The premises of this work were laid by the results of a large-scale RNAi screen performed by H. Grosshans and aimed at identifying new interaction partners of *let-7* in a unbiased manner (Ding et al. 2008). This initial experiment identified a potent genetic interaction between certain translation initiation factors, including subunits of eIF3, and *let-7,* which led me to propose the following thesis:

C. elegans miRNAs function *in vivo*, and at least partially, by inhibiting the initiation of translation on target mRNAs.

I explored this thesis by pursuing in parallel genetic as well as biochemical research approaches, which have been the objects of two independent publications included below (see sections 3.1. and 3.2.) (Ding et al. 2008; Ding and Grosshans 2009). The genetic experiments aimed at delineating the extent of the genetic interaction between *let-7* and the translation machinery in general, and translation initiation factors in particular. These experiments uncovered an unexpected widespread genetic interaction between *let-7* and the *C. elegans* translation machinery, suggesting that *let-7* activity and *let-7* targets are especially sensitive to alteration of the translation process. Additionally, these genetic data suggest, but do not demonstrate, that *let-7* activity may directly oppose that of eIF3 (Ding et al. 2008). The biochemical experiments aimed at directly assessing the translational status and mRNA level of endogenous target mRNAs in response to repression by miRNAs. The results demonstrated that miRNA-mediated repression is achieved in *C. elegans* by a combination of target mRNA degradation and inhibition of translation initiation and that the GW182 homologs AIN-1 and AIN-2 are essential mediators of these mechanisms (Ding and Grosshans 2009). Importantly, these observations bring early *C. elegans* studies, which argued for a post-initiation model (Olsen and Ambros 1999; Seggerson et al. 2002) into a new perspective. Additional experiments

aimed at deciphering the role of individual miRISC components in the repression mechanisms are also presented and discussed (see section 3.3.).

3. Results and discussion

3.1. Publication: "The *let-7* microRNA interfaces extensively with the translation machinery to regulate cell differentiation"

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Report

The *let-7* microRNA interfaces extensively with the translation machinery to regulate cell differentiation

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Key words: miRNA, let-7, translation factor, heterochronic, *C. elegans*, RNAi, eIF3, eIF6

MicroRNAs (miRNAs) are noncoding RNAs that regulate numerous target genes through a posttranscriptional mechanism and thus control major developmental pathways. The phylogenetically conserved *let-7* miRNA regulates cell proliferation and differentiation, thus functioning as a key regulator of developmental timing in *C. elegans* and a tumor suppressor gene in humans. Using a reverse genetic screen, we have identified genetic interaction partners of *C. elegans let-7*, including known and novel potential target genes. Initial identification of several translation initiation factors as suppressors of a *let-7* mutation led us to systematically examine genetic interaction between *let-7* and the translational machinery, which we found to be widespread. In the presence of wild-type *let-7*, depletion of the translation initiation factor eIF3 resulted in precocious cell differentiation, suggesting that developmental timing is translationally regulated, possibly by *let-7*. As overexpression of eIF3 in humans promotes translation of mRNAs that are also targets of *let-7*-mediated repression, we suggest that eIF3 may directly or indirectly oppose *let-7* activity. This might provide an explanation for the opposite functions of *let-7* and eIF3 in regulating tumorigenesis.

Introduction

MicroRNAs (miRNAs) are small, untranslated RNAs involved in numerous developmental pathways (reviewed in ref. 1). They function through an antisense mechanism where binding of an miRNA to complementary sequences in its target mRNAs ('cognate mRNAs') causes cognate mRNA repression, but the mechanisms of target mRNA repression are less clear. Many different, and sometimes contradictory, miRNA modes of action have been proposed (reviewed in refs. 2 and 3). These include inhibition of target mRNA translation either at the initiation or elongation step, target mRNA degradation in a non-endonucleolytic fashion, which may or may not result from deadenylation, and co-translational protein degradation. MicroRNAs may thus act through multiple mechanisms. These mechanisms may either function redundantly or as alternate pathways that affect only individual subsets of miRNAs and/or cognate mRNAs.[2,3]

The *C. elegans let-7* miRNA was originally identified as a component of the heterochronic pathway,[4] which controls the temporal fate of cells during postembryonic development (reviewed in ref. 5). Postembryonic development proceeds through the four larval stages, L1 through L4, followed by the sexually mature, adult stage. During normal development, cells adopt fates that are characteristic of the developmental stage of the animal, e.g., certain cells divide while others may exit the cell cycle and differentiate. Mutations in heterochronic genes may cause cells to prematurely adopt fates that are normally observed at a later developmental stage, i.e., cause precocious phenotypes. Alternatively, the mutant cells may display retarded phenotypes, i.e., characteristics typical of cells in earlier developmental stages. Partial loss of *let-7* activity causes retarded phenotypes, i.e., repetition of fourth larval stage (L4) cell fates, while more complete loss of activity causes animals to die by bursting through the vulva at the larval-to-adult transition.[4] These phenotypes are due to overexpression of *let-7* target genes and can be partially suppressed by knock-down of individual *let-7* target genes.[6-10]

let-7 is conserved in higher eukaryotes, with a striking 100% sequence identity in the case of the mature *let-7* of *C. elegans* and humans.[11,12] This, and the observation that *let-7* expression is temporally regulated in invertebrates as well as vertebrates,[11] suggests that *let-7* function may also be conserved. This view is supported by our recent finding that *C. elegans let-7* regulates *let-60/ras* expression, while human *let-7* regulates the *let-60* orthologue *RAS*.[7,13] Human *let-7* also regulates the chromatin-binding factor HMGA2, and failure of *let-7*-mediated HMGA2 repression promotes oncogenic transformation.[14-18] Consistent with overproliferation of cells with reduced *let-7* expression, *let-7* also represses the expression of the cell cycle regulator CDC25A (refs. 19 and 20). Reduced *let-7* expression in lung cancer[13,21] may contribute to tumorigenic transformation through upregulation of these oncogenes,[22,23] and reduced *let-7* expression levels are prognostic for poor patient survival.[21,24] *let-7* has also been shown to function as a tumor suppressor in breast cancer, where it controls proliferation and differentiation of tumor initiating cells.[25] The converging results from these different experimental systems have supported a model of *let-7* functioning as an important

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regulator of stem cell fates in both normal and tumor cells.[26] To achieve this function, *let-7* expression is highly regulated not only at the transcriptional level, but, as recent data suggest, also posttranscriptionally (reviewed in ref. 26).

To identify interaction partners of the *let-7* miRNA, which might include novel *let-7* targets, regulators of *let-7* expression, mediators of *let-7* activity, or heterochronic genes, we devised a high-throughput, functional genomics screen based on RNA interference (RNAi). Through this screen, we identified 41 known and novel interaction partners of *let-7*. As several genes directly or indirectly involved in translation were found among the novel *let-7* suppressors, we systematically examined genetic interactions between *let-7* and the core translational machinery and found them to be widespread. Consistent with translational control of the heterochronic pathway, we found that depletion of several of these genes, in particular subunits of the tumor promoting translation initation factor eIF3, caused abnormal timing of cell differentiation in the presence of wild-type *let-7*.

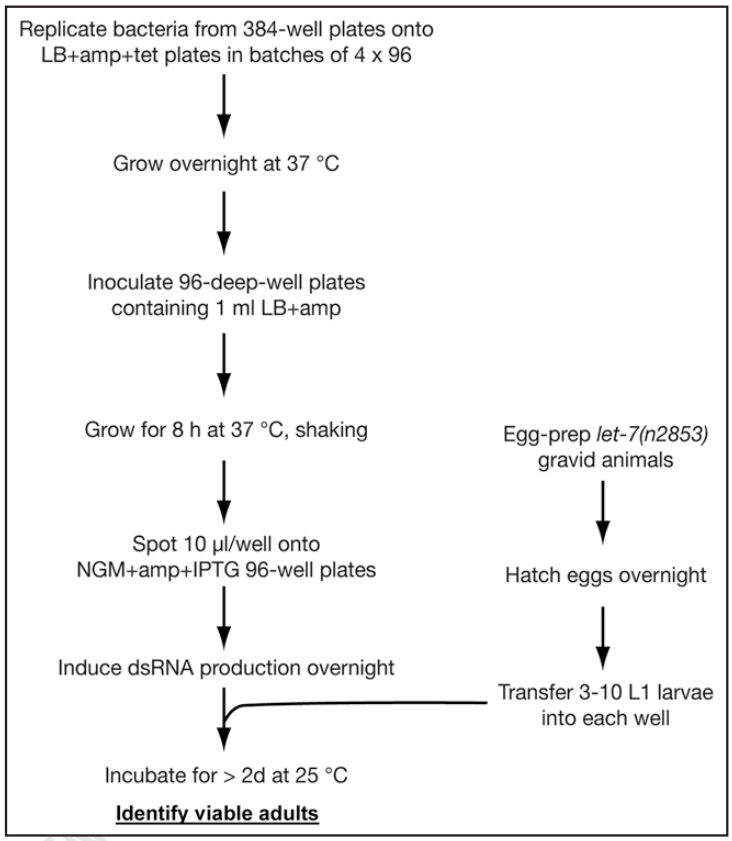

Figure 1. A high-throughput reverse genetics screen to identify suppressors of *let-7(n2853)* lethality. See main text and Materials and Methods for details.

Results

A reverse genetics screen reveals translation factors as suppressors of *let-7*. The temperature sensitive *let-7(n2853)* allele contains a point mutation in the mature *let-7* sequence that impairs target binding.[4,27] In addition, reduced accumulation of the mutant *let-7* RNA[28] further impairs target repression and as a result, mutant animals die by bursting through the vulva at the larval-to-adult transition when grown at or above 20°C (reviewed in ref. 4). RNAi-mediated knockdown of individual *let-7* targets can partially suppress this lethality.[6-10] To identify novel interaction partners of the *let-7* miRNA, we carried out a feeding RNAi screen to uncover additional suppressors of the *let-7(n2853)* bursting phenotype. We performed this screen by RNAi on synchronized L1 stage larvae to avoid missing factors whose efficient depletion would cause sterility and/or embryonic lethality. Using a previously described feeding library of bacteria producing double-strand RNA,[29] we individually tested the suppressing effect of RNAi on almost 90% of the genes on chromosome I, i.e., ca. 2,400 genes (Fig. 1). We found that 41 genes could efficiently suppress the conditional lethality of the *let-7* mutation when knocked down through RNAi by feeding (Table 1). Some but not all of the suppressor genes contained *let-7* complementary sites, as defined previously,[7] in their 3' untranslated regions (UTR) suggesting that these genes may be targets of the *let-7* RNA (Table 1).

Our screening procedure was validated by two observations. First, we blindly identified *lin-41*, the only known heterochronic gene in the chromosome I library, as a potent suppressor of *let-7(n2853)* when depleted. *lin-41* is a known downstream target of the *let-7* miRNA whose depletion had previously been shown to suppress *let-7(n2853)* (refs. 4 and 10). No RNAi construct targeting *lin-28*, another heterochronic gene and known suppressor of *let-7* encoded on chromosome I, was included in the RNAi library.[29] A second gene, *lss-4* was identified independently by us through a computational approach and also subsequently validated as a *let-7* target.[7] Second, seven genes in the library are targeted by two independent RNAi constructs,[29] and we identified both clones for four of these genes, *rpl-24.2*, *Y65B4BR.5*, *imb-5*/*xpo-2* and *spg-7*. In the remaining three cases differences in the RNAi phenotypes elicited by each pair of constructs were already previously noted.[29]

The largest class of suppressors identified in our screen is comprised of genes with a predicted function in the metabolism of RNA or protein, which account for nearly half (20/41) of the suppressors (Fig. 2). Genes from this category showed a 50% increase over the frequency found by Fraser et al.,[29] who queried the library for genes eliciting any phenotype when depleted in wild-type animals (Fig. 2). This observation may suggest that genes of this functional class are particularly important as targets and/or mediators of *let-7* function or are heterochronic genes. Given the tight posttranscriptional regulation of *let-7* expression,[26] it will also be of considerable interest to test in future work whether any of these novel *let-7* interactors control *let-7* maturation.

Our list of suppressors contained several translation initiation factors: two putative subunits of the eukaryotic translation initiation factor (eIF) 3, *eif-3.H* and *eif-3.E* (*C41D11.2* and *B0511.10*, respectively) and the delta subunit of eIF2B (*F11A3.2*). We also

Table 1 **Suppressors of *let-7(n2853)* lethality identified in a screen**

ORF[a]	Locus[b]	Function/Homologies[b,c]	LCS[d]	Suppression[e]
DNA synthesis				
W02D9.1	*pri-2*	DNA primase	-	+++
Y54E10A.15	*cdt-1*	*Hs* CDT1, *Dm* dup	-	+++
RNA metabolism				
B0511.6		DEAD box helicase, *Dm* pit	-	++
C17E4.5	*pabp-2*	Poly(A)-binding protein, *Hs* PABN1	-	++
C36B1.3	*rbp-3*	RNA polymerase II subunit	-	++
C53H9.2		Nucleolar GTPase, *Sc* Lsg1p	1	+
F14B4.3		RNA Polymerase I, second largest subunit	1	++
T19A6.2	*ngp-1*	Nucleolar GTPase, *Sc* Nog2p	-	+++
W01B11.3	*nol-5*	snoRNP associated, *Sc* Nop5p	1	++
W04A4.5		*Hs* Integrator isoform 1 (U1, U2 RNA processing)	-	+++
Y48G1A.4		snoRNP associated, *Sc* Nop14p	*nd*	++
Y54E10BR.6	*rbp-7*	RNA polymerase II subunit	-	++
Y71F9B.4	*snr-7*	*Hs* SNRPG (spliceosome subunit)	-	++
Y106G6H.2	*pab-1*	Poly(A)-binding protein, *Sc* Pab1p	-	++
Protein metabolism				
B0511.10	*eif-3.E*	Translational initiation factor eIF3 subunit	-	++
C03D6.8	*rpl-24.2*	Ribosomal protein L24-family, *Sc* Rlp24p	-	++
C12C8.3	*lin-41*	NHL domains	2	+++
C41D11.2	*eif-3.H*	Translational initiation factor eIF3 subunit	-	++
C47B2.5	*eif-6*	Translational initiation factor eIF6	-	++
Y47G6A.10	*spg-7*	AAA-ATPase, protease	-	++
F11A3.2[f]		Translational initiation factor, eIF2Bδ	-	+++
Y65B4BR.5		Nascent polypeptide associated complex α-chain	-	++
Energy/metabolism				
T09B4.9		Mitochondrial inner membrane translocase, *Hs* TIM44	-	++
W09C5.8		Subunit IV of cytochrome *c* oxidase	-	+
Chromosome dynamics				
C45G3.1	*aspm-1*	*Dm* Asp	-	+
T03F1.9	*hcp-4*	CENP-C homologue	-	++
Cell structure				
F56A3.3	*npp-6*	Nuclear pore protein	-	+++
T19B4.2	*npp-7*	Nuclear pore protein	2	+++
T21E12.4	*dhc-1*	Dynein heavy chain	-	++
Y48G1A.5	*imb-5*	*Sc* Cse1p, *Hs* CAS/CSE1	1	++
Y71F9AM.5	*nxt-1*	NTF2-family	-	+
Y105E8A.9	*apg-1*	γ-adaptin AP-1	1	++
H15N14.2	*nsf-1*	Vesicle fusion	-	+
Specific transcription				
C01G8.9	*lss-4*	*Dm* osa/eld	3	+++
F57B10.1		CREB/ATF family transcription factor	-	++
Signalling				
K12C11.2	*smo-1*	*Hs* SUMO-1	-	+
ZC581.1	*nekl-2*	NEK kinase family	-	++
Unknown				
F20G4.1	*smgl-1*	*Hs* Neuroblastoma amplified gene protein	-	++
F56A3.4	*spd-5*	Coiled coils	-	++
T23D8.3		Hs LTV1; in operon with eif-3C	-	++
Y63D3A.5	*tfg-1*	*Hs* TFG1 (TrkA-fused gene)	-	++

[a]Some ORFs were targeted by more than one dsRNA construct and/or construct names might differ from those of the target ORF indicated here. [b]Gene loci names according to Wormbase, Release 188. Gene names were not considered when assigning functional classes in cases where no published information or sequence homologies were available to support the gene designation. [c]*Sc: Saccharomyces cerevisiae, Dm: Drosophila melanogaster, Hs: Homo sapiens.* In most cases functions are predicted from homologies. [d]LCS: *let-7* complementary site as identified in[7]. *nd*: 3' UTR was not included in dataset used for LCS prediction. [e]+, >30%, ++, >50%, +++, >80% survival. [f]Sequencing revealed plasmid contained other than predicted insert, targeting the indicated ORF (chromosome V).

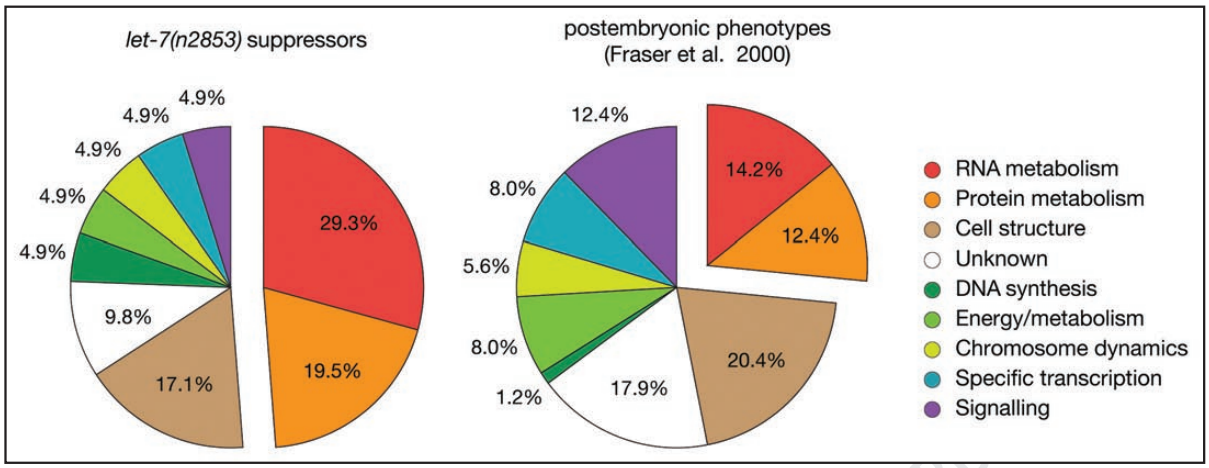

Figure 2. *let-7* suppressors are enriched for factors involved in RNA and protein metabolism. Genes involved in RNA and protein metabolism are enriched among the suppressors of the *let-7(n2853)* mutation. Indicated are the distributions across functional classes of genes causing suppression of the *let-7(n2853)*-associated lethality (left chart) or visible phenotypes in otherwise wild-type animals (right chart; assembled from data in ref. 29). Assignments to functional classes are from Wormbase Release WS188. Where gene assignments had changed from those used by Fraser et al., their data were adjusted accordingly. Note that both studies used an identical RNAi library.

identified *eif-6* (*C47B2.5*) as a *let-7* suppressor, an unexpected result given previous data showing that depletion of eIF6 abrogated miRNA target repression.[30] However, recent data from Drosophila S2 cells suggest that eIF6 may not be widely required for miRNA activity.[31,32]

As our initial screen did not cover the whole genome, we tested additional translation factors for a genetic interaction with *let-7*. Many translation factors have identifiable homologues in *C. elegans*[33] and we performed a systematic RNAi screen of these factors for suppression of the *let-7(n2853)* phenotype (Table 2). In most cases, knock-down of translation factors induced a slow growth or developmental arrest phenotype. We could frequently avoid developmental arrest by mixing bacteria that carried the dsRNA producing plasmid with those carrying a plasmid without insert. We found that additional translation factors could suppress the *let-7* mutation, in fact, most of the translation factors tested, including initiation, elongation, as well as termination factors, showed partial suppression. Many, but not all suppressors caused slow growth (Table 2), ruling out for at least a subset of translation factors that delayed development is the cause for *let-7* suppression. Moreover, an approximately wild-type rate of development also suggests that at least this subset of suppressors affects general translation only weakly. As a corollary, *let-7* function and/or the heterochronic pathway appear to be highly sensitive to altered translation levels.

Depletion of translation factors affects the heterochronic pathway. To ascertain that the suppression of the *let-7* vulval bursting phenotype through impaired translation was specific, i.e., mediated through a modulation of the heterochronic pathway rather than an indirect consequence of, for instance, aberrant vulval development, we investigated whether depletion of individual translation factors caused heterochronic phenotypes in the seam cells. Seam cells are a subset of hypodermal cells that display a stem-cell-like division pattern during larval stages. At the larval to adult transition, seam cells exit the cell cycle and fuse to form a syncytium, which subsequently secretes collagenous structures termed alae. *let-7* is expressed in the seam cells[34] and in its absence, seam cells fail to terminally differentiate at this time and instead divide again.[4] By contrast, overexpression of *let-7* causes the opposite, precocious phenotype and seam cells fuse after the L3-to-L4 molt.[4] We examined the effect of RNAi against *eif-3.B, eif-3.C, eif-3.E*, *eif-3.H* and *eif-6* in animals carrying a wild-type allele of *let-7*. We selected these factors because they were amongst the first suppressors we had identified. While seam cell fusion is not observed in mock-treated animals at early L4 stage, significant numbers of *eif-3(RNAi)* and *eif-6(RNAi)* animals displayed precocious seam cell fusion, as did the *lin-41(RNAi)* positive control animals (Fig. 3). In the case of *eif-3.B(RNAi)* and *eif-3.C(RNAi)* we again had to dilute the RNAi-inducing bacteria with inert bacteria to avoid developmental arrest of the affected animals. These findings directly demonstrate that knock-down of this subset of the suppressing translation factors causes heterochronic defects.

To obtain further evidence for a role of translation factors in the heterochronic pathway we analyzed the genetic interaction between *eif-3.E* or *eif-3.H* and *lin-41*. *lin-41* codes for a protein that prevents premature execution of adult fates by repressing production of the transcription factor LIN-29 until the L4 stage.[10] LIN-41 protein levels themselves are regulated through the interaction of *let-7* miRNA with the *let-7* complementary sites in the 3' UTR of *lin-41* mRNA.[4,10,27] *lin-41* loss-of-function mutations lead to partially penetrant precocious phenotypes in the seam cells and we previously showed that the penetrance of this phenotype can be enhanced when a second *let-7* interactor, the *let-7* target *daf-12*, is also knocked down.[7] Similarly, while only 53.1% of the *lin-41(ma104)* animals display precocious alae (±2.4% SEM), the penetrance of this phenotype was significantly ($p < 0.05$, student's t-test) increased to 65.0% ± 1.2% for *lin-41(ma104); eif-3.E(RNAi)* animals and 80.1% ± 0.4% for *lin-41(ma104); eif-3.H(RNAi)* animals.

Analysis of seam cell fusion and alae formation thus indicate that the translation factors investigated modulate the heterochronic

Table 2 **Genetic interactions between *let-7* and the translation machinery**

Translation factor	locus	ORF	*let-7(n2853)* suppression
Initiation factors			
eIF1		T27F7.3b	-
eIF1A[a]		H06H21.3	++
eIF2β[b]	*iftb-1*	K04G2.1	++
eIF2γ[d]		Y39G10AR.8	x
eIF2A		E04D5.1 (a, b)	-
eIF2Bα		ZK1098.4	-
eIF2Bβ		Y47H9C.7	++
eIF2Bγ	*ppp-1*	C15F1.4	++
eIF2Bδ		F11A3.2	+++
eIF2Bε[d]		D2085.3	+++
eIF3α[c,d]	*egl-45*	C27D11.1	x
eIF3b[c,d]	*eif-3.B*	Y54E2A.11a	+++
eIF3c[c,d]	*eif-3.C*	T23D8.4	+++
eIF3d[d]	*eif-3.D*	R08D7.3	++
eIF3e[d]	*eif-3.E*	B0511.10	+++
eIF3f[d]	*eif-3.F*	D2013.7	+++
eIF3g[d]	*eif-3.G*	F22B5.2	x
eIF3h	*eif-3.H*	C41D11.2	++
eIF3i[b]	*eif-3.I*	Y74C10AR.1	x
eIF3k	*eif-3.K*	T16G1.11	-
eIF3m[a]	*cif-1*	K08F11.3	+++
eIF4A[b,d]	*inf-1*	F57B9.6	x
eIF4A[a,d]		F57B9.3	+++
eIF4E-1+5	*ife-1* *ife-5*	F53A2.6 Y57A10A.30 (a, b)	-
eIF4E-2	*ife-2*	R04A9.4	-
eIF4E-3	*ife-3*	B0348.6 (a, b, c)	-
eIF4E-4	*ife-4*	C05D9.5	-
eIF4G[b,d]	*ifg-1*	M110.4 (a, b)	x
eIF4H	*drr-2*	T12D8.2	-
eIF5[b]		C37C3.2 (a, b, c)	++
eIF5A[d]	*iff-2*	F54C9.1	+++
eIF5B	*iffb-1*	Y54F10BM.2	+++
eIF6	*eif-6*	C47B2.5	++
Elongation factors			
eEF1A[b,d,e]	*eft-3* *eft-4*	F31E3.5 R03G5.1 (a, b, c, d)	++
eEF1B[e]		Y41E3.10 F54H12.6	++
eEF2[b,d]	*eft-2*	F25H5.4	+++
eEF2[c,d]	*eft-1*	ZK328.2	++
Release factors			
eRF1[c,d]		T05H4.6 (a, b)	x
eRF3[b,d]		H19N07.1	++

In some cases, RNAi titration was performed to overcome developmental block by mixing bacteria that carried the dsRNA producing plasmid with those carrying a plasmid without insert. [a]1:1 dilution; [b]1:5 dilution; [c]1:10 dilution; [d]slow growth; [e] multiple RNAi targets. Suppression: -,<20%, +, >20%, ++, >40%, +++, >80%, x, developmental block, n ≥ 60 worms for each. Control animals fed with bacteria carrying empty L4440 vector showed never more than 10% survival, whereas *daf-12(RNAi)*, our positive control, showed never less than 90% survival.

pathway. This shows that depletion of these factors does not simply superficially rescue the *let-7(n2853)* mutant phenotype but impacts on a pathway known to be regulated by *let-7*. These data would suggest that translation of one or several heterochronic genes, possibly *let-7* target genes, is inefficient and therefore particularly susceptible to further decreases in translation activity. Interestingly, overexpression of eIF3 subunits has been linked to various cancers (reviewed in ref. 35), and our findings indicate that the opposing effects of eIF3 and *let-7* on cell differentiation might be a contributing factor (see Discussion).

Discussion

We have identified several novel genetic interaction partners of the *let-7* miRNA. Some of these contain predicted *let-7* binding sites, and future work may establish them as bona fide *let-7* targets. Here, we have focused on the observation that our screen identified several translation initiation factors whose depletion allowed survival of *let-7(n2853)* worms (Table 1). Systematic depletion of individual translation factors subsequently allowed us to identify suppressors at each step of translation:[33] initiation, elongation and termination (Table 2). These suppressors include a subunit of eIF2, which is part of the eIF2/GTP/Met-tRNA$_i^{Met}$ ternary complex and all but one subunit of the eIF2B factor, which catalyzes guanine nucleotide exchange on eIF2 bound to GDP. Among the factors required to recruit the 40S ribosomal subunit to the ternary complex to form the 43S pre-initiation complex (PIC), we observed that eIF1A and all but one of the eIF3 subunits that permitted larval growth results are suppressors. The eIF4F complex, which comprises the cap-binding eIF4E, the scaffolding eIF4G, and the ATP-dependent RNA helicase eIF4A, recruits the 43S PIC to mRNA via an interaction between eIF3 and eIF4G. Among these factors, none of the eIF4E homologues (*ife-1* to *ife-5*) individually tested showed suppression, eIF4G and one homologue of eIF4A (*F57B9.3*) showed developmental arrest, whereas the eIF4A homologue encoded by the *F57B9.6* ORF showed potent suppression. Additionally eIF5, eIF5A and eIF5B were also identified as suppressors. Finally, all translation elongation and termination factors tested showed either a developmental block or suppression of the bursting phenotype.

Based on this result, it seems that suppression of the *let-7(n2853)* bursting phenotype can be rescued by decreasing the activity of virtually any step of the translation initiation process as well as the elongation and termination steps. Indeed, some of the other suppressors found in our screen are predicted, by homology, to be part of the ribosome (*rpl-24.2*) or function in its biogenesis (the putative snoRNP proteins W01B11.3 and Y48G1A.4 and the putative nucleolar GTPases homologous to yeast Lsg1p and Nog2p) and we also found two poly(A)-tail binding proteins, *pab-1* and *pabp-2*. However, not all translation factors could suppress *let-7* lethality. This may be due to redundancy (e.g., *ife-1* to *ife-5*), inefficient depletion by RNAi or a genuine lack of interaction between these two genes.

Unexpectedly, we also observed that depleting eIF6 rescues *let-7(n2853)* animals and causes precocious heterochronic phenotypes in the presence of wild-type *let-7*, although this factor was reported to be required for miRNA mediated repression.[30] If eIF6 were similarly required for *let-7* function, we would have expected to see the opposite phenotypes, i.e., enhancement of weak *let-7* alleles and or retarded heterochronic phenotypes. Our data would

thus argue against an involvement of eIF6 in *let-7* function, consistent with earlier reports from *D. melanogaster* cells that eIF6 does not seem to be generally involved in promoting miRNA function.[31,32]

It is also surprising to see that *eif-3.D*, along with almost all other eIF3 subunits, is found as an efficient suppressor. This observation is in contrast with a recent report indicating that *eif-3.D(RNAi)* in an RNAi sensitized strain enhanced the weak *let-7(mg279)* loss-of-function allele, as determined by increased vulval bursting.[36] However, we found that *eif-3.D(RNAi)* can induce vulval bursting even in wild-type animals where ca. 20% of animals die by bursting despite having functional *let-7*. It is possible that this bursting phenotype may dominate over a weak *let-7* allele, particularly when RNAi is performed in an RNAi sensitized strain, as done in the earlier report.[36]

We are particularly intrigued to see that depletion of eIF3 subunits causes precocious seam cell differentiation in the presence of wild-type *let-7*. This is because several of the thirteen subunits of human eIF3 have altered expression levels in cancers including lung, breast, cervical, esophageal, prostate and testicular cancers, and this aberrant expression is likely to contribute to oncogenesis.[35] For instance, INT6/eIF3e was originally identified as a common integration site of mouse mammary tumor virus,[37] and expression of the truncated INT6/eIF3e gene product, but not of the wild-type eIF3e gene, is sufficient to transform a number of cell lines.[38,39] Conversely, INT6/eIF3e loss-of-heterozygosity and decreased expression appear to be associated with breast and non-small cell lung cancers,[37] suggesting that INT6/eIF3e activity is particularly dosage dependent. Recently, eIF3h overexpression was shown to increase tumorigenic phenotypes in various cell lines,[40] and eIF3h was also found in a genome-wide association screen for loci conferring increased risk for colorectal cancer.[41] Finally, eIF3a, the largest eIF3 subunit, is overexpressed in human lung, breast, cervical and esophageal cancers,[42-45] and reduction of eIF3a levels in two human lung and breast cancer cell lines, respectively, is sufficient to suppress the malignant phenotypes in vitro.[46] eIF3a expression is also higher in fetal than in more differentiated tissues,[47] and thus reciprocal to *let-7* expression.[26]

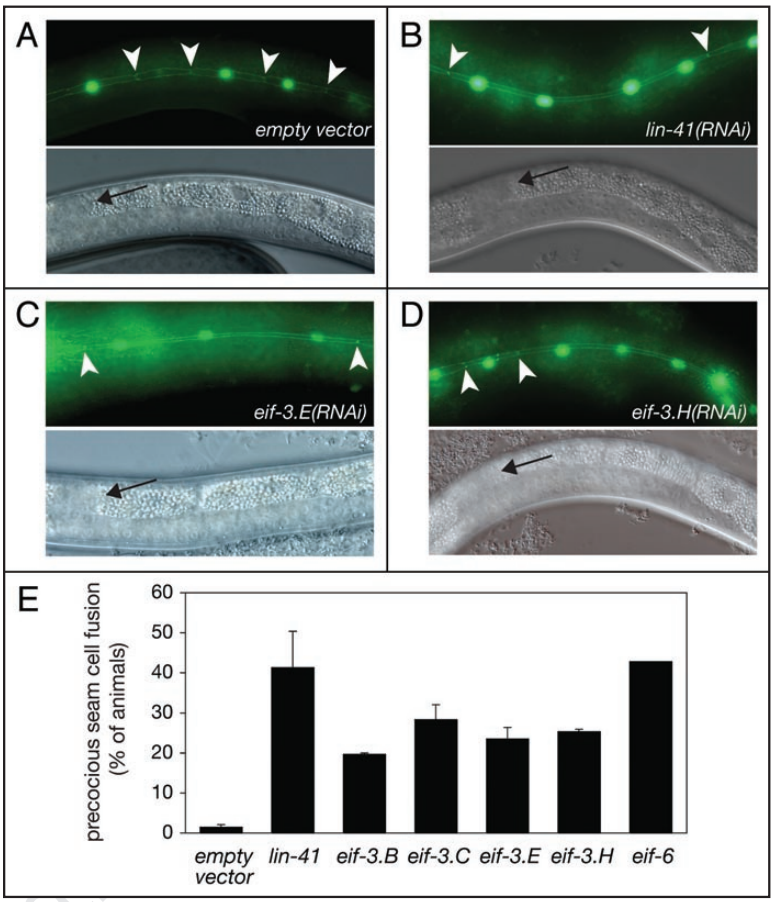

Figure 3. Reduced levels of eIF3-subunits cause precocious seam cell fusion. Synchronized *N2; wIs79* L1-stage larvae were grown on bacteria producing the indicated dsRNA and examined for presence of precocious seam cell fusion upon reaching early-L4 stage. (A–D) Photomicrographs of animals grown on the indicated bacteria. *Arrowheads* point to AJM-1/GFP signal between seam cells observed in the absence of cell fusion. *Arrows* in lower panels indicate the distal tips of the gonads, visualized through Nomarski optics, which demonstrate the appropriate early L4 developmental stage. Anterior of each animal is left, ventral down. Note that GFP and Nomarski micrographs show different parts of the same animal. (E) Percentages of animals with precocious seam cell fusion were averaged from at least two independent experiments. To avoid developmental arrest in the case of *eif-3.B* and *eif-3.C* subunits, and gonadal migration defects in the case of the *eif-3.E*, animals were fed bacteria expressing the appropriate dsRNA, diluted appropriately (1:2 to 1:6) with bacteria producing mock dsRNA. $n \geq 82$ for each. Error bars correspond to SEM.

As expression of eIF3 subunits in human cells appears to be highly coordinated[48] such that forced overexpression of individual subunits leads to increased accumulation of other subunits and incorporation into functional eIF3 complexes, it appears likely that additional eIF3 subunits are deregulated in tumors.

When viewed together with the fact that *let-7* functions as a tumor suppressor gene,[26] these findings suggest that the opposing roles of eIF3 and *let-7* on cell differentiation might be conserved beyond *C. elegans*. Indeed, increased amounts of eIF3 specifically stimulate translation of mRNAs involved in cell proliferation, in particular MYC and cyclin D1 (reviewed in ref. 48)—mRNAs that are repressed by *let-7*.[26] We might thus speculate that the opposing activities of eIF3 and *let-7* on a subset of cellular mRNAs contribute to the oncogenic functions of eIF3.

Taken together, we find widespread suppression of *let-7* loss-of-function through decreased cellular translation activity, suggesting that *let-7* targets or other heterochronic genes may be translationally regulated to allow proper timing of cell differentiation.

Materials and Methods

***let-7(n2853)* suppressor screen and RNAi.** Wild-type (N2*)* and *let-7(n2853)* (MT7626) strains used in this work were provided by the Caenorhabditis Genetics Center (CGC), which is founded by the NIH National Center for Research Resources. The screen was performed using RNAi by feeding with a published RNAi library[29] covering ca. 90% of the genes on *C. elegans* chromosome I. Additional RNAi clones were obtained from RNAi libraries,[49,50] or were created in the laboratory as follows by PCR on genomic DNA using the primers listed below. PCR fragments were digested with *Xba*I and *Kpn*I (pXD10, pXD11 and pXD12), *Nde*I/*Xho*I (pHG8) or *BamH*I/*Xho*I (pHG9) and ligated into L4440 (reviewed in ref. 51). The resulting constructs were transformed in *E. coli* HT115 for feeding RNAi experiments.

The screen was performed as illustrated in Figure 1, with every step done in duplicate. Supplements were used at the following concentrations: ampicillin: 100 μg/ml, tetracycline: 12.5 μg/ml, IPTG: 1 mM. Retesting and testing of additional translation factors was done at 20°C and 25°C on 6-cm diameter plates as described.[7] In some experiments, carbenicillin was used instead of ampicillin. Suppressor identity was confirmed through plasmid DNA recovery followed by sequencing. In some cases we were unable to stage worms reliably because RNAi caused gonad migration defects in the absence of oocytes. These candidates were discarded.

Enhancement of *lin-41(ma104)* precocious phenotypes was scored in at least two independent experiments with ≥19 animals per strain and a total of ≥100 animals scored per strain.

Oligonucleotide sequences.

Synthetic sequences are in lowercase, restriction sites underlined.

Name	5' to 3' sequence	Plasmid
eIF1_F1	gctctagagcTGAAGTTGCTCACCATCTCG	pXD10
eIF1_R1	ggggtaccccACTTCCTCGCCACTTCTTCA	(L4440-eIF1)
cif-1_F1	gctctagagcGATGATGTCAAGCAGCTCCA	pXD11
cif-1_R1	ggggtaccccCATTGTTCCGTTCCGAATCT	(L4440-cif-1)
eIF5B_F1	gctctagagcGAAGAGGATTCGGATGGTGA	pXD12
eIF5B_R1	ggggtaccccACCTCCTTCCTCTTGGCAAT	(L4440-eIF5B)
eif-3.B_F1	caccatATGGTCGAAATTGACTTTAAT	pHG8
eif-3.B_R1	tttctcgagTTAGTCTCTCATCTCCTCC	(L4440-eif-3.B)
eif-3.C_F1	tttggatccTGTCTCGCTTCTTCCATGC	pHG9
eif-3.C_R1	tttctcgagTTAGAAGGCTCGTGGCTTT	(L4440-eif-3.C)

Precocious seam cells fusion. Precocious seam cell fusion was analyzed using strain *wIs79*[*ajm-1::gfp; scm::gfp*] (ref. 9). Microscopy images were acquired using a Zeiss Axioplan microscope equipped with a Zeisscam CCD camera and Axiovision software. Images were cropped and levels adjusted using Adobe Photoshop software.

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3.2. Publication: "Repression of *C. elegans* microRNA targets at the initiation level of translation requires GW182 proteins"

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Repression of *C. elegans* microRNA targets at the initiation level of translation requires GW182 proteins

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MicroRNAs (miRNAs) repress target genes through a poorly defined antisense mechanism. Cell-free and cell-based assays have supported the idea that miRNAs repress their target mRNAs by blocking initiation of translation, whereas studies in animal models argued against this possibility. We examined endogenous targets of the *let-7* miRNA, an important regulator of stem cell fates. We report that *let-7* represses translation initiation in *Caenorhabditis elegans*, demonstrating this mode of action for the first time in an organism. Unexpectedly, although the *lin-4* miRNA was previously reported to repress its targets at a step downstream of translation initiation, we also observe repression of translation initiation for this miRNA. This repressive mechanism, which frequently but not always coincides with transcript degradation, requires the GW182 proteins AIN-1 and AIN-2, and acts on several mRNAs targeted by different miRNAs. Our analysis of an expanded set of endogenous miRNA targets therefore indicates widespread repression of translation initiation under physiological conditions and establishes *C. elegans* as a genetic system for dissection of the underlying mechanisms.

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Introduction

MicroRNAs (miRNAs) are small, untranslated RNAs involved in numerous developmental pathways in plants and animals (reviewed in Bushati and Cohen, 2007). They regulate a large fraction of cellular mRNAs by binding to complementary sequences in their target mRNAs ('cognate mRNAs'), but the mechanisms involved in subsequent repression of the mRNA are less clear (reviewed in Eulalio *et al*, 2008a; Filipowicz *et al*, 2008). In the best understood example, prevalent in plants, miRNAs function as small interfering (si)RNAs and induce mRNA cleavage through the RNA-induced silencing complex (RISC) when binding to perfectly complementary sites in their target mRNAs (Jones-Rhoades *et al*, 2006). In animals, this appears to be the exception, as most animal miRNAs are only partially complementary to their targets (Bushati and Cohen, 2007), thus precluding RISC-mediated cleavage. Early work on the *Caenorhabditis elegans lin-4* miRNA established, instead, the paradigm that miRNAs functioned by translationally repressing their targets at a step downstream of translation initiation, without substantially affecting transcript levels (Olsen and Ambros, 1999; Seggerson *et al*, 2002). By contrast, recent studies aimed at recapitulating miRNA function in cell-free systems concluded that miRNAs inhibit target mRNA translation at the initiation step (Wang *et al*, 2006; Mathonnet *et al*, 2007; Thermann and Hentze, 2007; Wakiyama *et al*, 2007). Inhibition of translation initiation, as evidenced by the hallmark shift of target mRNAs from heavy to light polysomal or monosomal fractions of sucrose density gradients in response to the miRNA, has also been observed in a number of cell-based studies. However, such studies also identified additional and sometimes conflicting miRNA modes of action (Eulalio *et al*, 2008a; Filipowicz *et al*, 2008). These mechanisms include inhibition of target mRNA translation after initiation, target mRNA degradation in a non-endonucleolytic manner, which may or may not result from deadenylation, and co-translational protein degradation. Target mRNA degradation has also been observed for some miRNA targets *in vivo*, in *C. elegans* and zebrafish (Bagga *et al*, 2005; Giraldez *et al*, 2006).

Only a single study has so far demonstrated regulation of an endogenous mRNA, CAT1, by its cognate miR-122 miRNA at the level of translation initiation (Bhattacharyya *et al*, 2006). The other studies that examined endogenous miRNA targets instead provided evidence against repression of translation initiation (Olsen and Ambros, 1999; Seggerson *et al*, 2002; Kong *et al*, 2008), and this includes the only two studies that have tested this mechanism in an animal model, under physiological conditions (Olsen and Ambros, 1999; Seggerson *et al*, 2002). It is currently unclear whether this divergence of results denotes specific mechanisms operating for individual miRNAs and/or targets. Alternatively, the transfected miRNA target reporters that were used in the bulk of studies showing repression of translation initiation by miRNAs might be particularly conducive to this mode of action, consistent with reports that transfection modalities (Lytle *et al*, 2007) and choice of the promoters that drive reporter gene expression (Kong *et al*, 2008) can affect the apparent mode of target repression.

Consistent with the elusive nature of miRNA mechanism(s), few molecular players have been identified. Mature miRNAs occur in a complex with Argonaute (AGO) family proteins, and it has been suggested that direct binding of AGO to the mRNA cap may be responsible for miRNA target repression (Kiriakidou *et al*, 2007), but this has been

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controversial (Eulalio *et al*, 2008b). The translation initiation factor eIF6 has been identified as a component of a large AGO2-containing complex in human cells and eIF6 depletion was shown to impair miRNA target gene silencing in human cells and *C. elegans* (Chendrimada *et al*, 2007). However, it has been suggested that the involvement of eIF6 may be indirect (Filipowicz *et al*, 2008), and studies of *Drosophila* cells have indicated that eIF6 may not be generally required for miRNA function (Eulalio *et al*, 2007, 2008b). Consistent with the latter notion, depletion of *C. elegans* eIF6 appears to enhance rather than diminish *let-7* miRNA activity by genetic criteria (Ding *et al*, 2008).

AGO proteins also bind to members of the GW182 protein family in various organisms and this interaction contributes to miRNA function (reviewed in Ding and Han, 2007). Tethering of GW182 to an mRNA leads to degradation of this mRNA, and, conversely, GW182 depletion impairs miRNA activity (Liu *et al*, 2005; Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2008b). In *C. elegans*, combined loss of the two GW182-like proteins AIN-1/-2 partially phenocopies loss of the AGO proteins ALG-1/-2 and causes upregulation of reporter genes under miRNA control (Ding *et al*, 2005; Zhang *et al*, 2007). The level and extent to which AIN-1/-2 contribute to miRNA function have remained unknown, although it has been suggested that they might localize repressed miRNA targets to P-bodies to enable their degradation (Ding *et al*, 2005).

We have focused here on the *C. elegans let-7* miRNA to examine the mechanism of action of miRNAs *in vivo*. *let-7* was originally identified as a component of the *C. elegans* heterochronic pathway (Reinhart *et al*, 2000), which controls the temporal fate of cells during postembryonic development. Several *let-7* target genes have been identified (Slack *et al*, 2000; Abrahante *et al*, 2003; Lin *et al*, 2003; Grosshans *et al*, 2005; Lall *et al*, 2006) and among these, *lin-41* and *daf-12* have been characterized most extensively and their *let-7*-binding sites partially mapped (Reinhart *et al*, 2000; Slack *et al*, 2000; Vella *et al*, 2004; Grosshans *et al*, 2005). This availability of *in vivo* validated targets combined with the fact that the sequence of *let-7* is perfectly conserved in animals (Pasquinelli *et al*, 2000; Lagos-Quintana *et al*, 2002), and that it has been used to examine miRNA mechanisms of action in diverse experimental systems (Bagga *et al*, 2005; Pillai *et al*, 2005; Nottrott *et al*, 2006; Mathonnet *et al*, 2007; Wakiyama *et al*, 2007), makes *let-7* particularly suitable for our analysis. In addition, understanding the mode of action of this specific miRNA is of particular interest because of its important developmental and pathological functions as a potent regulator of stem cell fates and a tumour suppressor (reviewed in Büssing *et al*, 2008).

We report that *let-7* causes repression of translation initiation as well as degradation of its endogenous *lin-41* and *daf-12* target mRNAs. Other miRNAs silence their targets by the same mechanisms, and this includes *lin-4* miRNA, previously reported to repress translation at a level after initiation (Olsen and Ambros, 1999; Seggerson *et al*, 2002). Translational repression requires the GW182 proteins AIN-1/-2, as does mRNA degradation. Our findings indicate that downregulation of translation initiation is widely used under physiological conditions in *C. elegans* and establish the nematode as a system for genetic dissection of this process.

Results

Translational blockade of endogenous let-7 target genes

We recently observed widespread genetic interaction between *let-7* and the translational machinery in *C. elegans* (Ding *et al*, 2008). These findings prompted us to examine whether *let-7* regulates its targets translationally *in vivo*. To this end, we fractionated whole animal lysates by sucrose density gradient ultracentrifugation to analyse the polyribosome association of endogenous *let-7* targets in wild-type and *let-7(n2853)* mutant *C. elegans* at the L3 developmental stage, when *let-7* activity is low, and at the late L4 stage, when *let-7* activity is high (Reinhart *et al*, 2000) (Figure 1A; Supplementary Figure S1). As the two *let-7* targets *daf-12* and *lin-41* (Slack *et al*, 2000; Grosshans *et al*, 2005) are expressed at very low levels in L4 stage larvae (Snow and Larsen, 2000; Bagga *et al*, 2005 and this study, below), we used reverse transcription–quantitative PCR (RT–qPCR) to quantify them. It is to be noted that all experiments were performed using random hexamer oligonucleotides to prime RT, to include even mRNA, the poly(A) tail of which might be short due to the action of the miRNA (Eulalio *et al*, 2008a; Filipowicz *et al*, 2008). Additional control experiments, described below, further confirmed that we are detecting full-length mRNAs rather than partially stable degradation fragments.

We found that both *lin-41* and *daf-12* mRNAs were moderately, but consistently depleted from the highly translated polysomal fractions in wild-type relative to *let-7* mutant animals at the late L4 stage (Figure 1B and C; Supplementary Figure S1), in agreement with decreased translation initiation (Eulalio *et al*, 2008a). By contrast, *ama-1* and *act-1* mRNAs, which are not targeted by *let-7*, displayed similar translational profiles in both strains (Figure 1B and C; Supplementary Figure S1).

L3 stage animals express little or no *let-7* (Reinhart *et al*, 2000); accordingly, we see no difference when comparing polysomal association of *daf-12* and *lin-41* mRNAs between *let-7* mutant and wild-type animals at this stage (Figure 1B and C; Supplementary Figure S1). Moreover, in wild-type animals, polysome association of *daf-12* and *lin-41* is decreased at L4 compared with L3 stage, consistent with the establishment of an inhibitory mechanism affecting translation initiation as *let-7* expression starts. A more moderate decrease of polysome association is also seen when performing this comparison for *let-7* mutant animals, suggesting that the *n2853* allele may provide residual *let-7* activity or that alternative mechanisms, possibly the *let-7* sister miRNAs *mir-48*, *mir-84* and *mir-241* (Abbott *et al*, 2005; Li *et al*, 2005), may contribute.

To exclude the possibility that the RNA that we analysed in our sucrose gradients was not representative for the total pool of cellular RNA, we performed the following control experiments. We used TRIzol to extract total RNA directly from ground worms, from the cleared lysate used for sucrose gradient centrifugation and from the pellet left behind upon lysate clearing. We found, first, that ~90% of the RNA is in the lysate supernatant and will thus be loaded on the sucrose gradient. Second, composition of RNA in the supernatant and pellet is comparable, neither *let-7* target nor control mRNAs are preferentially enriched in, or depleted from, the supernatant relative to RNA retained in the pellet (Supplementary Figure S2). Finally, although increasing sucrose concentration

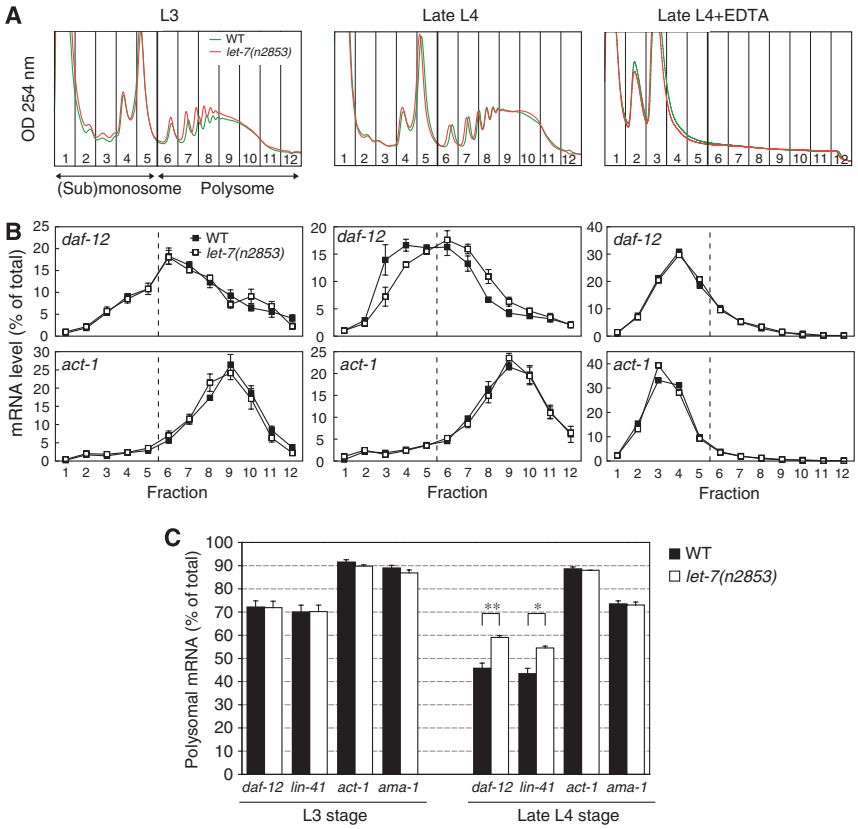

Figure 1 *let-7* decreases translation initiation on *daf-12* and *lin-41* mRNAs. (**A**) Typical polysome profile of wild-type and *let-7(n2853)* animals with or without EDTA treatment. (**B**) Distribution of *daf-12* and *act-1* mRNAs across polysome profiles from synchronized wild-type and *let-7(n2853)* animals at the L3 and late L4 stage, with or without EDTA treatment. (**C**) Polysomal fraction of *daf-12*, *lin-41*, *ama-1* and *act-1* mRNAs in L3 and late L4 wild-type and *let-7(n2853)* animals as a percentage of the total ($*P<0.05$, $**P<0.01$). mRNA levels were analysed by RT–qPCR. EDTA treatment was performed in duplicate, one representative experiment is shown. All other panels in this and subsequent figures show the averages of $n \geqslant 3$ independent experiments. For this and all subsequent figures, 'WT' denotes the wild-type *N2* strain, and error bars are s.e.m.

in total lysates decreased the yield of extracted RNA, RNA composition was largely unaffected (Supplementary Figure S2). To ensure comparable recovery from each fraction and greatest possible reproducibility, we equalized sucrose concentration in all fractions to 30% (w/v) prior to RNA extractions in all our experiments. This set of control experiments confirms that any results that we obtained in our analysis can be considered representative of the total pool of cellular RNA.

To determine further whether the fast-sedimenting mRNA was indeed associated with polyribosomes, we treated lysates with EDTA and observed that all four mRNAs were shifted to the top of gradients. Distributions became indistinguishable for late L4 wild-type and *let-7(n2853)* animals (Figure 1; Supplementary Figure S1). As EDTA also disrupts non-ribosomal ribonucleoprotein complexes, we further used puromycin to disassemble specifically polysomes by inducing premature termination of the elongating peptide chains. Puromycin treatment of extracts collapsed polysomes and shifted the mRNAs deeper into the gradient (Supplementary Figure S3), possibly reflecting aggregation of the mRNA and not further pursued by us. The resulting sedimentation patterns were indistinguishable for wild-type and *let-7* animals and occurred for *let-7* target as well as control mRNAs. The coincident loss of polysomes and shift of mRNAs demonstrates that our assay examines mRNAs associated with translation-competent ribosomes.

We conclude from these data that *let-7* depletes its targets *lin-41* and *daf-12* from *bona fide* polysomes, consistent with blocking translation initiation on these mRNAs.

Translational repression requires let-7 complementary sites in the lin-41 3′ UTR

The *lin-41* 3′ untranslated region (3′UTR) is necessary and sufficient to confer *let-7*-mediated regulation on an unrelated reporter gene (Slack *et al*, 2000). To verify that *let-7* impaired *lin-41* translation by binding to the *lin-41* 3′UTR, we employed transgenic animals expressing a *lacZ* reporter gene fused to the *lin-41* 3′UTR or a mutant variant thereof lacking the *let-7*-binding sites (Figure 2A). We expressed the transgene from the *col-10* promoter to accumulate it specifically in the seam cells, where *let-7* mediates *lin-41* repression (Slack *et al*, 2000; Johnson *et al*, 2003).

Consistent with inhibition of translation initiation, we observed that only 40% of the reporter mRNA was associated with polysomes in wild-type animals, whereas this level reached almost 70% in *let-7* mutant animals. Deletion of

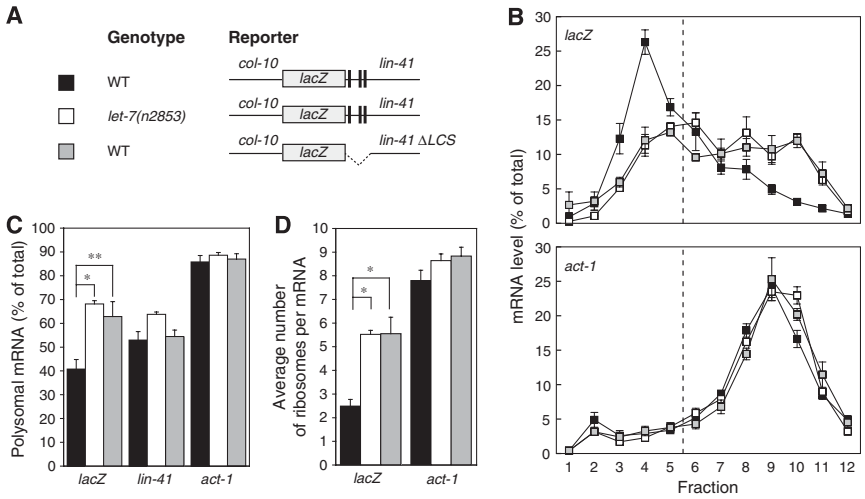

Figure 2 *lin-41* translational repression is mediated through *let-7*-binding sites. (**A**) Schematic representation of the reporter strains. Black square, WT *N2*; *Is[col-10*::*lacZ*::*lin-41]*, white square, *let-7(n2853)*; *Is[col-10*::*lacZ*::*lin-41]*, grey square, WT *N2*; *xeIs11[col-10*::*lacZ*::*lin-41-ΔLCS]*. The vertical lines in the *lin-41* 3′UTR represent *let-7* complementary sequences (LCSs). (**B**) *lacZ* and *act-1* mRNA distributions, determined by RT–qPCR, across polysome profiles. (**C**) Polysomal fraction of *lacZ*, *lin-41* and *act-1* mRNAs as a percentage of the total. (**D**) Average number of ribosomes on *lacZ* and *act-1* mRNA (*$P<0.05$, **$P<0.01$). Synchronized late L4 reporter animals were used. Note that the repression of endogenous *lin-41*, carrying the full-length 3′UTR, is maintained in the wild-type strain expressing the truncated *col-10*::*lacZ*::*lin-41-ΔLCS* transgene. The difference between endogenous *lin-41* translational repression in *let-7(n2853)* and wild-type animals is no longer statistically significant ($P=0.067$) in transgenic animals, possibly due to sequestering of endogenous *let-7* by reporter transgenes in wild-type animals.

the validated *let-7*-binding sites in the reporter 3′UTR (Vella *et al*, 2004) relieved translational repression to the same extent in wild-type animals (Figure 2B and C). Consequently, *let-7* mutation or deletion of its binding sites increased the average number of ribosomes per *lacZ* mRNA by more than two-fold relative to the wild-type situation, whereas the average number of ribosomes per *act-1* mRNA stayed constant (Figure 2D; see Materials and methods for details on the calculation). This result shows that the interaction between *let-7* and its binding sites in the *lin-41* 3′UTR mediates significant translational repression of the target mRNAs. This is confirmed by our finding that loss of *let-7* regulation causes a $\geqslant$5-fold derepression of the *lacZ* reporter (Supplementary Figure S4), although mRNA levels change less than two-fold (see below).

mRNA degradation does not correlate with translational repression

Bagga *et al* (2005) observed dramatic reduction of target mRNA levels in the presence of their cognate miRNAs in *C. elegans*. By RT–qPCR, we determined mRNA levels of the *let-7* targets in total RNA that we prepared from aliquots of the same whole animal lysates that were used for the polysome profile experiments (Figure 3A). At the L3 stage, *lin-41* and *daf-12* mRNA levels are similar in wild-type and *let-7(n2853)* animals. However, at the late L4 stage, *lin-41* mRNA is six-fold and *daf-12* two-fold more abundant in *let-7(n2853)* relative to wild-type animals. Similar ratios were obtained when summing up the amounts of these mRNAs across all fractions of the sucrose gradients, further confirming that RNA extracted from the gradients is representative of total cellular full-length mRNAs. It is to be noted that the levels of *lin-41* and *daf-12* mRNAs are reduced by two-fold even in the *let-7* mutant animals between the L3 and L4 stages.

For *lin-41*, our results are in agreement with those seen by Bagga *et al* (2005), and northern blot analysis of total RNA using a probe against *lin-41* identified a single band, the intensity of which mirrored the signal obtained by RT–qPCR in the same backgrounds (Figure 3B). Although *lin-41* mRNA levels in individual sucrose gradient fractions were below the limit of detection by northern blot analysis, these results essentially exclude the possibility that accumulation of *lin-41* mRNA degradation products could bias our RT–qPCR results and confirm that we reliably quantify full-length mRNAs.

For *daf-12* mRNA, its low abundance prevents detection by northern blotting even in unfractionated, total RNA without prior selection of polyadenylated mRNA (Snow and Larsen, 2000). Therefore, to confirm that our RT–qPCR assay similarly measures the levels of full-length *daf-12* mRNA, we tested a second set of primers, and obtained comparable results as expected (Supplementary Figure S5A). Finally, we examined the expression levels of both *daf-12* and *lin-41* using cDNA obtained through oligo-dT-primed RT. Again, we obtained comparable results (Supplementary Figure S5B and C), arguing against the detection of a stable degradation product and suggesting that any residual poly(A) tail on these mRNAs is sufficient to support priming through oligo-dT oligonucleotides. In summary, we confirm by several independent methods that our assays quantify full-length mRNAs, and we find that the *daf-12* and *lin-41* mRNAs are not only translationally repressed by *let-7* but also subject to degradation.

Translational repression of *daf-12* is at least equal to that of *lin-41* but the decrease of *daf-12* mRNA levels is more modest, suggesting that translational repression and transcript degradation may not be directly linked. Indeed, although the *lacZ*::*lin-41* reporter mRNA is very efficiently

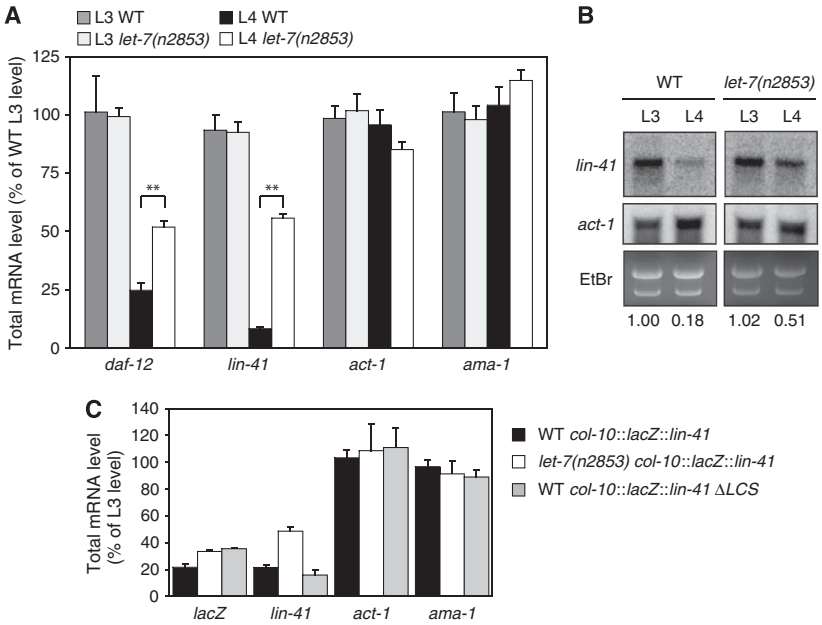

Figure 3 *let-7* mediates target mRNA degradation. (**A**) Analysis of *daf-12*, *lin-41*, *ama-1* and *act-1* total mRNA levels by RT–qPCR. Data are normalized for the average of *ama-1* and *act-1* values (**$P<0.01$). (**B**) Northern blot analysis of *lin-41* and *act-1* mRNA levels using 20 μg of total RNA. Numbers indicate *lin-41* mRNA levels normalized for *act-1*. Wild-type and *let-7(n2853)* samples are presented in separated panels for clarity; however, RNA samples were assessed on the same membrane and exposition time is identical. (**C**) Analysis of *lacZ*, *lin-41*, *act-1* and *ama-1* total mRNA levels in L4 stage reporter animals by RT–qPCR, data normalized for the average of *act-1* and *ama-1* values.

repressed translationally, mRNA levels differed by less than two-fold in wild-type relative to *let-7(n2853)* animals (Figure 3C). Although these findings strongly argue against a scenario where lower abundance of an mRNA diminishes its access to the translational machinery, we wished to exclude the possibility further that the translational effects that we observed were due to altered mRNA levels. *ugt-63* and *vit-1* are differentially expressed in synchronized late L4 wild-type and *let-7(n2853)* animals but are not direct targets of *let-7* (B Hurschler and HG, unpublished data). Although *vit-1* was four-fold less abundant in *let-7(n2853)* than in wild-type animals, and *ugt-63* was two-fold more abundant, the translational profiles of both genes were similar in wild-type and *let-7(n2853)* (Supplementary Figure S6). Thus, altered mRNA levels *per se* do not appear to influence the efficiency of translation initiation.

Multiple miRNAs function by preventing translation initiation

The finding that *let-7* mediates repression of translation initiation on its targets in *C. elegans* was unexpected, as *C. elegans lin-4* was previously reported to repress these mRNAs at a step downstream of translation initiation (Olsen and Ambros, 1999; Seggerson *et al*, 2002). To determine whether repression of translation initiation is specific for *let-7* or a more general mechanism, we tested whether *lin-4* repressed translation initiation of *lin-14* and *lin-28*, two experimentally validated targets (Wightman *et al*, 1993; Moss *et al*, 1997). *lin-4* is first expressed in the mid-L1 stage and represses *lin-14* by late L1/early L2 and *lin-28* one stage later (Olsen and Ambros, 1999; Seggerson *et al*, 2002). When we compared extracts from late L2 stage wild-type and *lin-4(e912)* mutant animals, we observed that both mRNAs were shifted into the polysomal fraction in the mutant (Figure 4A and B). This shift is particularly pronounced for *lin-28*, where the effect is highly statistically significant (Figure 4A). By contrast, polysome association of the control mRNAs *act-1* and *ama-1* and the *let-7* target *daf-12* is identical in *lin-4(e912)* and wild-type animals (Figure 4A and B). We conclude that *lin-4*, similar to *let-7*, can repress its target at the level of translation initiation.

lin-14 and *lin-28* transcript levels are increased in *lin-4* mutants compared with wild-type animals, whereas *daf-12*, *act-1* and *ama-1* mRNA levels remain unchanged (Figure 4C). The observation that *lin-4* induces a stronger translational blockade of *lin-28* than of *lin-14* and conversely a more pronounced degradation of *lin-14* than of *lin-28* further suggests that translational repression and target mRNA degradation are not directly linked mechanisms.

Translational repression and degradation of miRNA targets require the GW182 proteins AIN-1 and AIN-2

Having established that miRNAs mediate both target mRNA degradation and translational repression *in vivo*, we sought to identify the factors mediating these mechanisms. Good candidates were the GW182 homologues AIN-1 and AIN-2 (Ding *et al*, 2005; Zhang *et al*, 2007), as depletion of GW182 causes upregulation of miRNA target genes in various systems (Ding and Han, 2007). However, although mRNA degradation is readily prevented upon GW182 depletion, derepression of those miRNA targets that are not strongly regulated by degradation is typically well below that seen with AGO depletion (Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2007), consistent with the proposal that GW182 proteins might

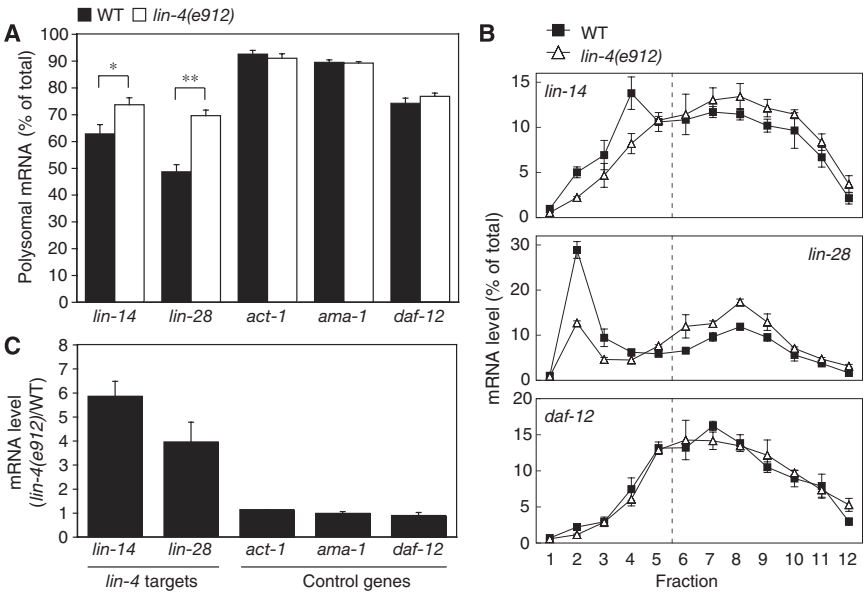

Figure 4 *lin-4* inhibits translation initiation of *lin-14* and *lin-28*. (**A**) Polysomal fraction of *lin-14*, *lin-28*, *act-1*, *ama-1* and *daf-12* in synchronized late L2 wild-type and *lin-4(e912)* animals as a percentage of the total (*$P<0.05$, **$P<0.01$). (**B**) *lin-14*, *lin-28* and *daf-12* distribution across polysome profiles from synchronized late L2 wild-type and *lin-4(e912)* animals. (**C**) Analysis of total mRNA levels in synchronized late L2 wild-type and *lin-4(e912)* animals by RT–qPCR, data are normalized for the average of the control gene values.

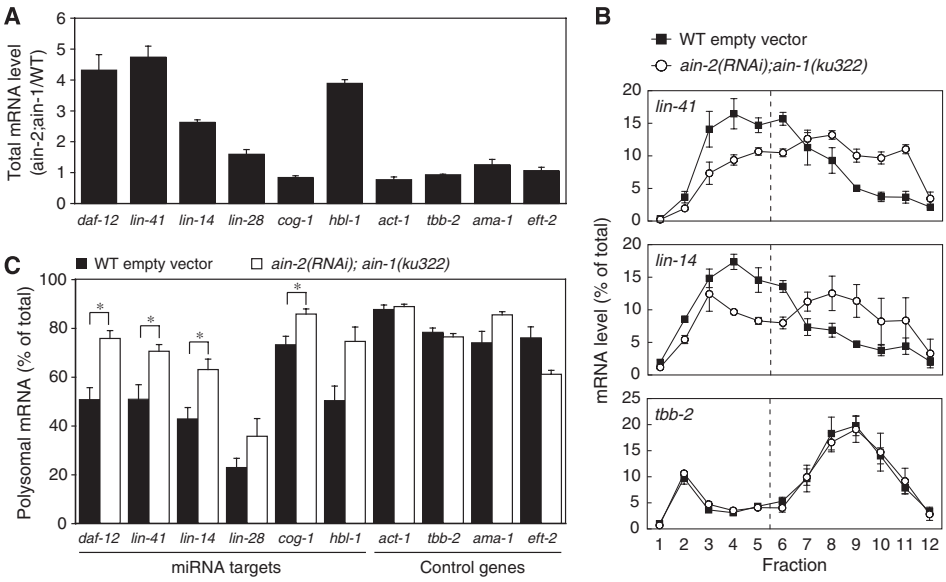

Figure 5 AIN-1 and AIN-2 mediate translational repression and degradation of miRNA target mRNAs. (**A**) Analysis of total mRNA levels in synchronized late L4 wild-type and *ain-2(RNAi); ain-1(ku322)* animals by RT–qPCR, data are normalized for the average of the control gene values. (**B**) *lin-41*, *lin-14* and *tbb-2* distribution across polysome profiles from synchronized late L4 wild-type and *ain-2(RNAi); ain-1(ku322)* animals. (**C**) Polysomal fraction of several miRNA targets and control genes in synchronized late L4 wild-type and *ain-2(RNAi); ain-1(ku322)* animals as a percentage of the total (*$P<0.05$).

enhance miRNA activity by targeting repressed mRNAs to P-bodies for degradation (Ding *et al*, 2005).

To determine whether depletion of the GW182 family members AIN-1 and AIN-2 permitted uncoupling of translational repression and degradation of miRNA targets, we performed polysome profile analyses on L4 stage wild-type and *ain-2(RNAi); ain-1(ku322)* animals and analysed various targets of multiple miRNAs: the *let-7* targets *daf-12* and *lin-41*, the *lin-4* targets *lin-14* and *lin-28*, the *lsy-6* targets *cog-1* (Johnston and Hobert, 2003) and *hbl-1*, which is targeted by *mir-48*, *mir-84*, *mir-241*, *let-7* and *lin-4* (Abrahante *et al*, 2003; Lin *et al*, 2003; Abbott *et al*, 2005). As predicted,

depletion of AIN-1/-2 increased *lin-41* and *daf-12* transcript levels (Figure 5A). However, to our surprise, translational repression of both *let-7* targets was also efficiently relieved (Figure 5B and C). In fact, the relief of both modes of *let-7* target repression was more extensive in *ain-2; ain-1* than in *let-7(n2853)* mutant animals, possibly suggesting that remaining *let-7* activity or distinct miRNAs, perhaps of the *let-7* family, contribute to residual repression of *lin-41* and *daf-12* in *let-7(n2853)* animals. Consistent with this idea, mRNA levels of the two *let-7* targets *daf-12* and *lin-41* are upregulated in *miR-48 miR-241; miR-84* triple mutant animals (Supplementary Figure S7).

Translational repression was also relieved for *lin-14*, *lin-28*, *cog-1* and *hbl-1*, although the results for *lin-*28 and *hbl-1* narrowly missed statistical significance (*lin-28*, $P = 0.053$; *hbl-1*, $P = 0.056$). We also analysed genes not known to be miRNA targets (*act-1*, *tbb-2*, *ama-1* and *eft-2*). We observed no effect on total mRNA levels and no consistent trend of translational upregulation in response to AIN-1/-2 depletion (Figure 5). Low abundance of the investigated miRNA target mRNAs in late L4 wild-type animals (see Figure 3B) prevented us from performing northern blot analysis on polysome profile fractions. However, consistent results were obtained by RT–qPCR with multiple *lin-14* primer pairs (Supplementary Figure S8) and by semiquantitative classical RT–PCR (Supplementary Figure S9) confirming our observation that translational repression of miRNA target is relieved in AIN-1/-2 depleted animals.

Taken together, these data reveal that translational control is a mechanism that is widely used by miRNAs *in vivo*. Equally significant, our results show that AIN-1/-2 have a general and important function in the process. Notably, although transcript levels of *lin-14*, *lin-28* and *hbl-1* increased in *ain-2; ain-1* mutant relative to wild-type animals, *cog-1* mRNA levels remained unchanged (Figure 5C), demonstrating that translational repression can occur independently of target mRNA degradation.

Discussion

We report here that endogenous *daf-12* and *lin-41* mRNAs are translationally controlled by *let-7 in vivo*. Polysomal shifts can even be seen in whole worm lysates, despite the fact that *let-7* regulates these targets only in a subset of those tissues where they are expressed (Antebi *et al*, 2000; Slack *et al*, 2000; Johnson *et al*, 2003). Nonetheless, the degree of spatial and temporal co-expression of the miRNA and its targets limits the sensitivity of our assay, as demonstrated for *pha-4*, a third experimentally validated *let-7* target (Grosshans *et al*, 2005). *let-7* regulates *pha-4* in the intestine (Grosshans *et al*, 2005), but not in the pharynx, where *pha-4* expression is particularly strong, and where *let-7* is not co-expressed (Azzaria *et al*, 1996; Johnson *et al*, 2003). Under these conditions, we can neither observe polysomal shifts (Supplementary Figure S1) nor *pha-4* mRNA accumulation (data not shown) in *let-7* mutant relative to wild-type animals. By contrast, the magnitude of repression of translation initiation can be well appreciated for the *col-10*::*lacZ*::*lin-41* reporter mRNA, which is exclusively expressed in the seam cells where *let-7* is also active.

We subsequently expanded our studies to mRNAs targeted by other miRNAs, including the *lin-4* targets *lin-14* and *lin-28* and found that these, too, were repressed at the level of translation initiation. These findings resonate well with results from cell-free and a subset of cell culture-based assays (Eulalio *et al*, 2008a; Filipowicz *et al*, 2008), and extend these studies by demonstrating such function under physiological conditions. Equally important, most published evidence for translation initiation has been obtained through the use of transfected target reporter genes, the mode of repression of which appears to be susceptible to transfection conditions (Lytle *et al*, 2007) and promoter choice (Kong *et al*, 2008). To our knowledge, only a single endogenous target gene was demonstrated to be repressed by this mechanism (Bhattacharyya *et al*, 2006), whereas this has been ruled out for others (Olsen and Ambros, 1999; Seggerson *et al*, 2002; Kong *et al*, 2008). Our study now demonstrates that repression of translation initiation is nonetheless widespread for endogenous miRNA targets, and different miRNAs.

Our finding that *lin-4* represses its targets, at least in part, at the level of translation initiation contrasts with earlier experiments that had revealed largely unchanged polysomal distributions of *C. elegans lin-14* and *lin-28* before (L1 stage) and after (L2 stage) the onset of expression of their cognate miRNA, *lin-4* (Olsen and Ambros, 1999; Seggerson *et al*, 2002). The reason for this discrepancy is currently unclear, but as *lin-4* mutant animals were not compared with wild-type animals in the earlier studies, it is possible that the resulting translational profiles might also have reflected developmental, *lin-4*-independent effects such as potential repression of *lin-28* by LIN-66 (Morita and Han, 2006). We also note that at least one of the earlier publications (Olsen and Ambros, 1999) displayed a—statistically nonsignificant—trend of *lin-14* shifting to the (sub-) monosomal fraction at the L2 stage, when *lin-4* expression is high.

Other miRNAs, in addition to *lin-4*, possibly its 'sister' *mir-237*, or even *let-7* (Reinhart *et al*, 2000; Slack *et al*, 2000; Esquela-Kerscher *et al*, 2005; Grosshans *et al*, 2005; Morita and Han, 2006), may also regulate *lin-14* or *lin-28*, and contribute to the polysomal shift observed in *ain-2; ain-1* mutant animals. However, although this remains to be tested for *lin-28*, we did not detect any change in *lin-14* mRNA levels or translation in response to the *let-7(n2853)* mutation (data not shown). Irrespective of this possibility, our experiments using *lin-4* mutant animals clearly demonstrate that this miRNA can mediate repression of translation initiation.

In most instances, we observed significant amounts of cognate mRNA degradation alongside translational repression, but the extent of degradation varied by target. Moreover, there was no clear correlation between the extent of translational repression and target mRNA degradation, for example, we observed more degradation for endogenous *lin-41* than for the reporter mRNA, although less translational silencing is apparent for the endogenous transcript. Although we cannot formally rule out the possibility that this specific case reflects differences between endogenous targets and targets expressed from transgenes or that transcriptional effects mediated by the *lin-41* promoter may contribute to these differences, we favour the idea that translational repression and target mRNA degradation may be independent mechanisms and that seam cells favour translational repression. This is consistent with an earlier study showing that different types of cultured cells evoke different responses to

identical target mRNA reporters, with degradation dominating in some cell lines and translational blockade in others (Schmitter *et al*, 2006). Indeed, the observation that *cog-1* is regulated translationally, but not at the level of mRNA degradation, further supports our conclusion that mRNA degradation and translational repression are two distinct mechanisms *in vivo* that may, however, frequently act together on the same miRNA targets.

It is to be noted that our results cannot rule out that the two mechanisms might function sequentially in that translational repression precedes mRNA degradation (e.g. Selbach *et al*, 2008). Indeed, we note that repression of translation initiation by *lin-4* appears more prominent for *lin-28* than *lin-14*. As *lin-14* is repressed at an earlier developmental stage than *lin-28* (Olsen and Ambros, 1999; Seggerson *et al*, 2002), it is tempting to speculate that increased *lin-14* mRNA degradation might deplete the monosomal pool of translationally repressed mRNA, effectively reducing the apparent polysomal shift. Analysis of polysome profiles at increased temporal resolution might help to address this possibility in the future.

Early reports on GW182 suggested a more auxiliary function in miRNA activity, with a greater importance in repressing targets susceptible to mRNA degradation (Ding *et al*, 2005; Liu *et al*, 2005; Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2007). However, as AIN-1/-2 appear rather distantly related to GW182 proteins (Behm-Ansmant *et al*, 2006), it was unknown whether their functions can be inferred from GW182 activity in other organisms. Moreover, recent work on *Drosophila* GW182 has shown that degradation-independent, possibly translational, repressive mechanisms may also crucially involve GW182 (Eulalio *et al*, 2008b), possibly in an miRNA- and/or target-specific manner. We find that depletion of the GW182 proteins AIN-1/-2 severely impairs both cognate mRNA degradation and translational control *in vivo*, for a number of different miRNA targets and miRNAs, supporting the notion that these proteins are widely used, essential effectors of miRNA activities. This conclusion is also consistent with the *alg-1/2*-like phenotypes observed in *ain-2; ain-1* double mutant animals (Zhang *et al*, 2007). In view of the fact that both mRNA degradation and translational repression require AIN-1/-2, we speculate that GW182 proteins may coordinate these two activities, possibly through interaction with distinct mediators or effectors, the identities of which remain to be elucidated.

Taken together, our study provides insights into miRNA function in an animal model and establishes *C. elegans let-7* as a model for the genetic dissection of miRNA-mediated repression of translation initiation, complementing available biochemical systems. The fact that translational repression *in vivo* may be substantial at least for a subset of targets, and possibly occur even without any degradation altogether, suggests that the identification of targets of this important miRNA will benefit greatly from recently established proteomics approaches (Baek *et al*, 2008; Selbach *et al*, 2008).

Materials and methods

C. elegans strains and RNAi

Wild-type N2, MT7626: *let-7(n2853)*, MH2385: *ain-1(ku322)* and DR721: *lin-4(e912)* strains were provided by the CGC; CT5a: *N2;Is[goa-1*::*gfp; col-10*::*lacZ*::*lin-41]* (Caudy *et al*, 2003) by R Plasterk; VT1066: *mir-48 mir-241(nDf51); mir-84(n4037)* (Abbott *et al*, 2005) by V Ambros. The HW211: *let-7(n2853);Is[goa-1*::*gfp; col-10*::*lacZ*::*lin-41]* strain was obtained by crossing CT5a with MT7626. The HW390: *N2;xeIs11[rol-6(su1006); col-10*::*lacZ*::*lin-41ΔLCS 1-3]* strain was generated by genomic integration of an extrachromosomal array made of pFS1031 and *rol-6(su1006)* (Vella *et al*, 2004), followed by several rounds of backcrossing to *N2*. As *ain-2(tm1863); ain-1(ku322)* (Zhang *et al*, 2007) double mutant animals grow very poorly, we exposed *ain-1(ku322)* animals to *ain-2(RNAi)*, starting with L1 larvae and using a published RNAi feeding construct and protocol (Kamath *et al*, 2003; Grosshans *et al*, 2005). Enhancement of the alae defect phenotype from 21% penetrant in the *ain-1(ku322)* single mutant to 100% in the double mutant confirmed efficient *ain-2* knockdown ($n \geqslant 17$ each). To circumvent reduced brood size associated with the *lin-4(e912)* allele and obtain a sufficiently large synchronized population, *lin-4(e912)* animals were expanded at 20°C on *lin-14(RNAi)*. Following synchronization by hatching in M9 buffer, animals were shifted back to standard non-RNAi food (OP50) and grown to the desired stage for extract preparation. The reappearance of the *lin-4(e912)* phenotypes (long, egg-laying defective) on these animals excluded the possibility that *lin-14(RNAi)* had prolonged effects.

Polysome profile analysis

A detailed description can be found in the Supplementary data. Briefly, lysates of synchronized worms were layered on linear sucrose gradients (15–60% w/v) and centrifuged for 3 h at 39 000 r.p.m., 4°C, using a SW-40 rotor and an Optima L-80 XP Ultracentrifuge (Beckman Coulter). The gradients were fractionated in 12 fractions of equal volume while absorbance at 254 nm was recorded. The entire gradient was fractionated so that any pelleted material would be recovered with the last fraction. However, we typically found very little RNA in this fraction, suggesting that RNAs did not substantially occur in heavy particles or compartments under our experimental conditions. After adjusting sucrose concentration in each fraction to 30% (w/v), RNA was extracted using TRIzol (Invitrogen) and RNA integrity confirmed on ethidium bromide-stained agarose gels before proceeding to RT. RNA recovery was quantitative under these conditions reaching up to 89% of input RNA. See Results section for a discussion of control experiments confirming that RNA extracted from the lysate is representative of the composition of total cellular RNA.

RT–qPCR

RNA RT was performed using the ImProm-II™ Reverse Transcription System (Promega) with random hexamer primers, according to the manufacturer's recommendations using equal amounts of RNA (400 or 800 ng) for each sample to avoid saturating the RT reactions in fractions with high concentrations of RNA. For polysome profile fractions, relative transcript levels quantified by qPCR (below) were subsequently corrected for the total amount of RNA extracted from each fraction and expressed as a percentage of the total amount recovered for the gradients. Identical results were obtained using oligo-$(dT)_{15}$ primers and equal volumes of RNA without applying any correction, validating the method and establishing that qPCR following RT by random hexamer is unlikely to detect stable degradation fragments. This was also directly examined for *lin-14* and *daf-12* using distinct qPCR primer sets, which yielded comparable results to the original primers.

qPCR reactions were performed in technical duplicate using the ABsolute™ QPCR SYBR® Green ROX Mix (Thermo Fisher Scientific), according to the manufacturer's recommendations, on an ABI Prism 7000 Sequence Detection System coupled to ABI Prism 7000 SDS 1.0 Software (Applied Biosystems). Relative transcript levels were calculated using the $2^{-\Delta C_t}$ method (Livak and Schmittgen, 2001). For all primer pairs (Supplementary data), amplification efficiencies were determined to be equal or superior to 1.8. Control reactions lacking either the reverse transcriptase or template mRNA confirmed specificity of the amplification reaction.

Northern blot

RNA electrophoresis and transfer were performed as described earlier (Bagga *et al*, 2005). UV crosslinked membranes were hybridized using ULTRAhyb hybridization buffer (Ambion) according to the manufacturer's recommendations with randomly radiolabelled probes prepared from PCR-amplified DNA (see Supplementary data for oligonucleotide sequences). Radioactive

signals were detected and quantified using a Storage Phosphor Screen and a Typhoon 9400 with the Imagequant TL software (all GE Healthcare).

Calculation of average number of ribosomes per mRNA

To calculate the average number of ribosomes per mRNA, each gradient fraction was assigned an average number of ribosomes by counting the peaks of the polysome profile at 254 nm. This number was multiplied with relative amount of the mRNA detected in this specific fraction. The sum of this product over all the fractions yielded the average number of ribosomes per mRNA.

Statistical methods

All statistical significances were calculated using the paired one-tailed Student's *t*-test. Note that the statistical significance in this stringent test not only depends on the average and standard deviation of the data sets but also on the variation of the difference between the paired values so that error bars will not fully reflect the statistical significance obtained through this test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Supplementary information

Supplementary Material and Methods

Polysome profile analysis

Synchronized worms were grown at 25 °C (N2 L3 and late L4, *let-7(n2853)* and *ain-2(tm1863);ain-1(RNAi)*) or at 20 °C (N2 L2 and *lin-4(e912)*) and on NGM (2%) plates seeded with *E. coli* OP50. L2 (~200'000), L3 (~50'000), or late L4 (~30'000) worms, staged by vulval and gonad development, were harvested and washed three times with cold M9 supplemented with 1 mM cycloheximide and once with Lysis Buffer (see composition below) without RNasin and PTE/DOC. Worms were pelleted and frozen in liquid N_2. Worms resuspended in 450µl of cold Lysis Buffer (20mM Tris pH 8.5, 140 mM KCl, 1.5 mM $MgCl_2$, 0.5 % Nonidet P40, 2% PTE (polyoxyethylene-10-tridecylether), 1% DOC (sodiumdeoxycholate monohydrate), 1mM DTT, 1mM cycloheximide, 0.4 U/µl RNasin) were crushed to a fine powder using mortar and pestle precooled with liquid N_2. As the powder thawed, lysates were collected and cleared by centrifugation (10 min. at 10'000 g, 4 °C).

Lysate absorbances at 260 nm were measured and equivalent amounts of material were loaded on sucrose gradients and centrifuged for 3 hours at 39'000 rpm, 4 °C, using a SW-40 rotor and an Optima™L-80 XP Ultracentrifuge (Beckman Coulter). Linear 15% to 60% (w/v) sucrose gradients were prepared from 15% (w/v) and 60% (w/v) sucrose solutions containing 20 mM Tris pH 8.5, 140 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and 1 mM cycloheximide using a Gradient Master (Biocomp).

Gradient fractionation was performed using a Tris Pump (Teledyn ISCO), a Gradient Fractionator (BR-184-X, Brandel), and a fraction collector (FC-203B, Gilson). Absorbance profiles were recorded at 254 nm with an Econo UV monitor EM-1 (Biorad) coupled to a data acquisition device (DI-158U, DATAQ Instruments) using the WinDaq Serial Acquisition software (version 3.17). Gradients were fractionated in 12 fractions of equal volume. RNA from lysates and from each fraction was extracted with TRIzol (Invitrogen), according to the manufacturer's recommendations. RNA aliquots were quality controlled on ethidium bromide stained agarose gels prior to RT-qPCR analysis. For EDTA treatment, cycloheximide was omitted in M9, lysis buffer and sucrose solution and 10 mM EDTA was added to the lysis buffer and sucrose solutions. Incubation of extracts with puromycin (5mM for 20 min. at 37 °C) led to collapse of the polysomes, confirming their translational competence. Unexpectedly, all mRNAs, including those not known to be regulated by miRNAs, shifted towards dense sucrose gradients under these conditions, suggesting that the ribosome-free RNAs aggregated.

β-galactosidase assay

CT5a, HW211 and HW390 animals were grown on DH5α bacteria that lack β-galactosidase activity. Two animals of the desired stage were transferred into 1.5 µl BGA buffer (50 mM potassium phosphate, pH 7.2, 1 mM $MgCl_2$) and fixed and permeabilized by addition of 15 µl ice-cold acetone. Animals were resuspended in 8 µl of CPRG staining solution (1.5 mM CPRG [chlorophenolred-ß-D-galactopyranoside] in BGA buffer) and incubated for 1 h to 24 h at 37 °C. Aliquots of 2 µl were measured on a Nanodrop spectrophotometer at $\lambda = 575$ nm and corrected for absorption at $\lambda = 700$ nm. Enzyme activities remained stable over the whole period as determined by constant changes in extinction per hour and worm measured

for different time points. No difference in β-galactosidase activity was seen for transgenic animals grown at either 20 °C or 25 °C; animals lacking *lacZ* transgenes did not exhibit any β-galactosidase activity. Correct stages were confirmed by subsequent DAPI staining of the animals used for the assay and microscopic analysis of the gonad.

Classical PCR and quantification

Classical RT-PCRs were performed on cDNA prepared as mentioned in the experimental procedures section of the main text. PCRs were performed using Taq DNA polymerase (Qiagen) according to the manufacturer's recommendations. *lin-14*, *lin-41*, and *tbb-2* mRNA level were determined using same set of primers as for RT-qPCR reactions (qPCR lin-14 F2/R2, qPCR lin-41 F/R, qPCR tbb-1 F1/R1, respectively). PCR products were resolved on SyberSafe (Invitrogen) stained agarose gels. Quantification of the PCR products was performed by densitometry analysis of agarose gel pictures using the ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Oligonucleotide sequences

Oligonucleotides used in the present study. The qPCR CT5a F1 and R1 primers were used to detect the *lacZ::lin-41* reporter mRNAs. The forward primer is complementary to the lacZ sequence and the reverse primer to the lin-41 3'UTR.

Name	5' to 3' sequence
qPCR act-1 F1	GTTGCCCAGAGGCTATGTTC
qPCR act-1 R1	CAAGAGCGGTGATTTCCTTC
qPCR ama-1 F1	GGATCGAAGGGATCGAAGA
qPCR ama-1 R1	TGGAAGAAGAATTCCGATGG
qPCR daf-12 F2	GATCCTCCGATGAACGAAAA
qPCR daf-12 R2	CTCTTCGGCTTCACCAGAAC
qPCR daf-12 F3	TTATATCCCGGCCACTCTCA
qPCR daf-12 R3	TGGAACACCAGGTAACGACA
qPCR lin-41 F1	GGATTGTTCGACACCAACG
qPCR lin-41 R1	ACCATGATGTCAAACTGCTGTC
qPCR CT5a F1	CGGTCGCTACCATTACCAAC
qPCR CT5a R1	CTGGAATGTGTGTGCTTTGC
qPCR pha-4 F1	CATGCAAGGAGGAGGAATTT
qPCR pha-4 R1	TCGTGAGTTCTTGGCCTTG
qPCR vit-1 F1	GAGGTTCGCTTTGACGGATA
qPCR vit-1 R1	GGCTTCACATTCCTCGTTCT
qPCR ugt-63 F1	AAAGACCCCCTGGATTGAAG
qPCR ugt-63 R1	TCTCTTTGATGAGCCAAGCA
qPCR tbb-2 F1	CAAATTCTGGGAGGTCATCTC
qPCR tbb-2 R1	CATACTTTCCGTTGTTGGCT
qPCR eft-2 F1	TGTGTTTCCGGAGTGTGTGT
qPCR eft-2 R1	CCATCGTCGTCTCCGTAAGT
qPCR hbl-1 F1	ACTGCACATATGCCACCAAA
qPCR hbl-1 R1	TGATGTAACCGGCTCAACTG
qPCR cog-1 F1	TCCAGCACTCAATGCAACTC
qPCR cog-1 R1	TTTTGTACGACGGTTTTGGA
qPCR lin-14 F1	TGCAAATCTTCCAATCAAAGG
qPCR lin-14 R1	TTCTGCCTGAGCCTCTTCTC
qPCR lin-14 F2	GGATTCAATGCGACAGGATT
qPCR lin-14 R2	CGATGCTGGTTTCAATGATG
qPCR lin-28 F1	ATTCAAGAGCGATCGAATGG
qPCR lin-28 R1	CACACTTTTGCATCGGTTTTT

NB lin-41 F1	CAAGACTCCTTTCGGTGCTC
NB lin-41 R1	CTGCACGGCTCATCAAAGTA
NB act-1 F1	GTTGCCCAGAGGCTATGTTC
NB act-1 R1	CAAGAGCGGTGATTTCCTTC

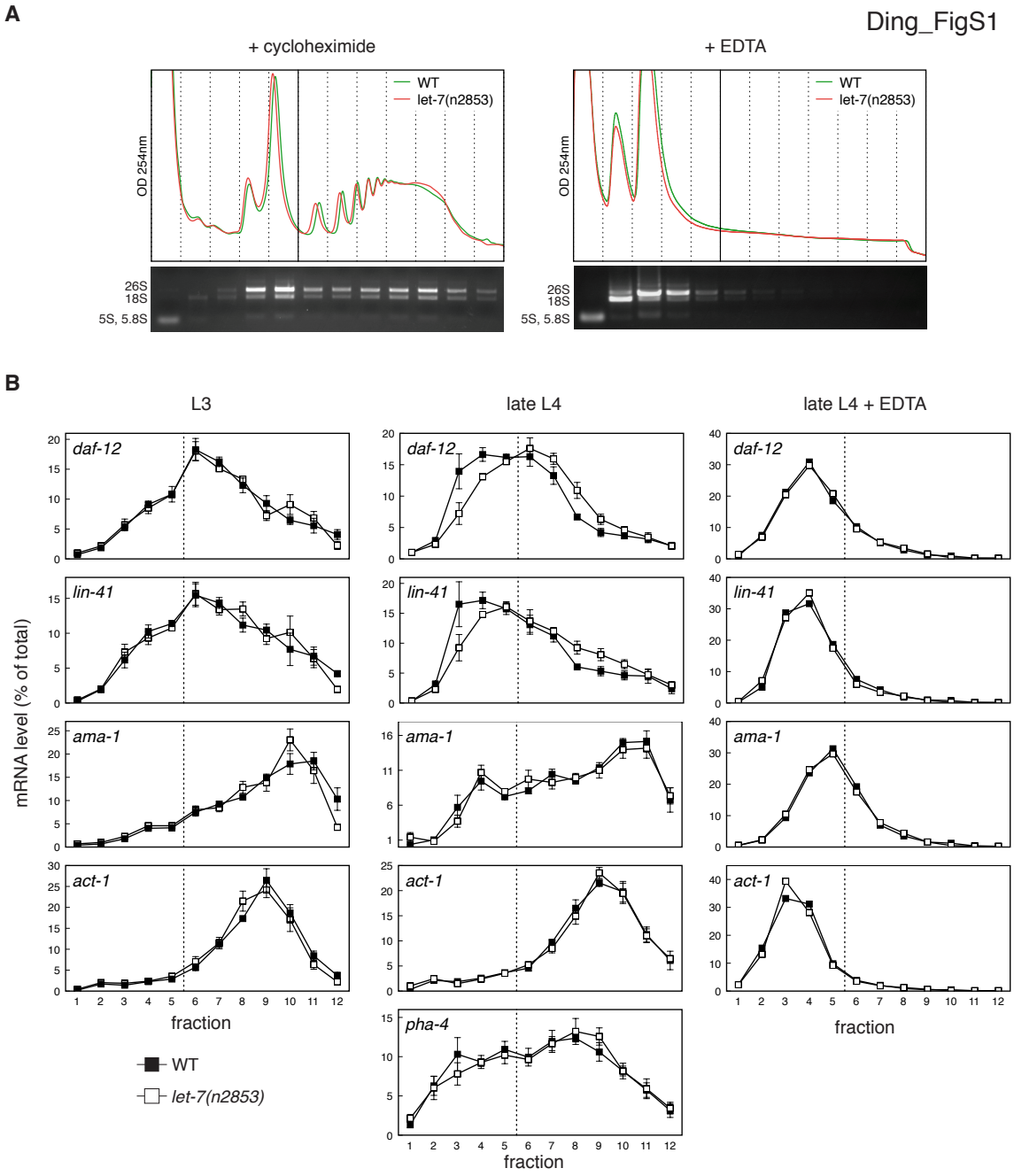

Figure S1: Polysome profile distribution of endogenous *let-7* target mRNAs.
(**A**) Typical polysome profiles from synchronized late L4 wild-type and *let-7(n2853)* animals in the presence of cycloheximide or EDTA, respectively. The solid line represents the separation between the (sub)monosomal and polysomal fractions. Lower panels are ethidium bromide stained agarose gels of RNA extracted from polysome profile fractions. The 26S, 18S, 5.8S and 5S rRNAs are visible. As indicated by absorbance profiles at 254 nm and rRNA distributions across fractions, cycloheximide treatment preserves polysome integrity, whereas EDTA treatment induces ribosome dissociation into 40S and 60S ribosomal subunits. Note that the absorbance profiles are those also shown in Fig. 1 (**B**) Distribution of the *let-7* targets *daf-12* and *lin-41* and of the control genes *ama-1* and *act-1* across polysome profiles from synchronized wild-type and *let-7(n2853)* L3 animals treated with cycloheximide, late L4 animals treated with cycloheximide, and late L4 animals treated with EDTA. *pha-4* distribution is shown for synchronized wild-type and *let-7(n2853)* late L4 animals only. The dotted line represents the separation between the (sub)monosomal and polysomal fractions. Panel B, except EDTA treatment, shows averages of at least three biological replicates. EDTA treatment was performed in duplicate, one representative experiment is shown. Error bars are SEM. *daf-12* and *act-1* data are as in Fig. 1 and included for comparison.

Ding_FigS2

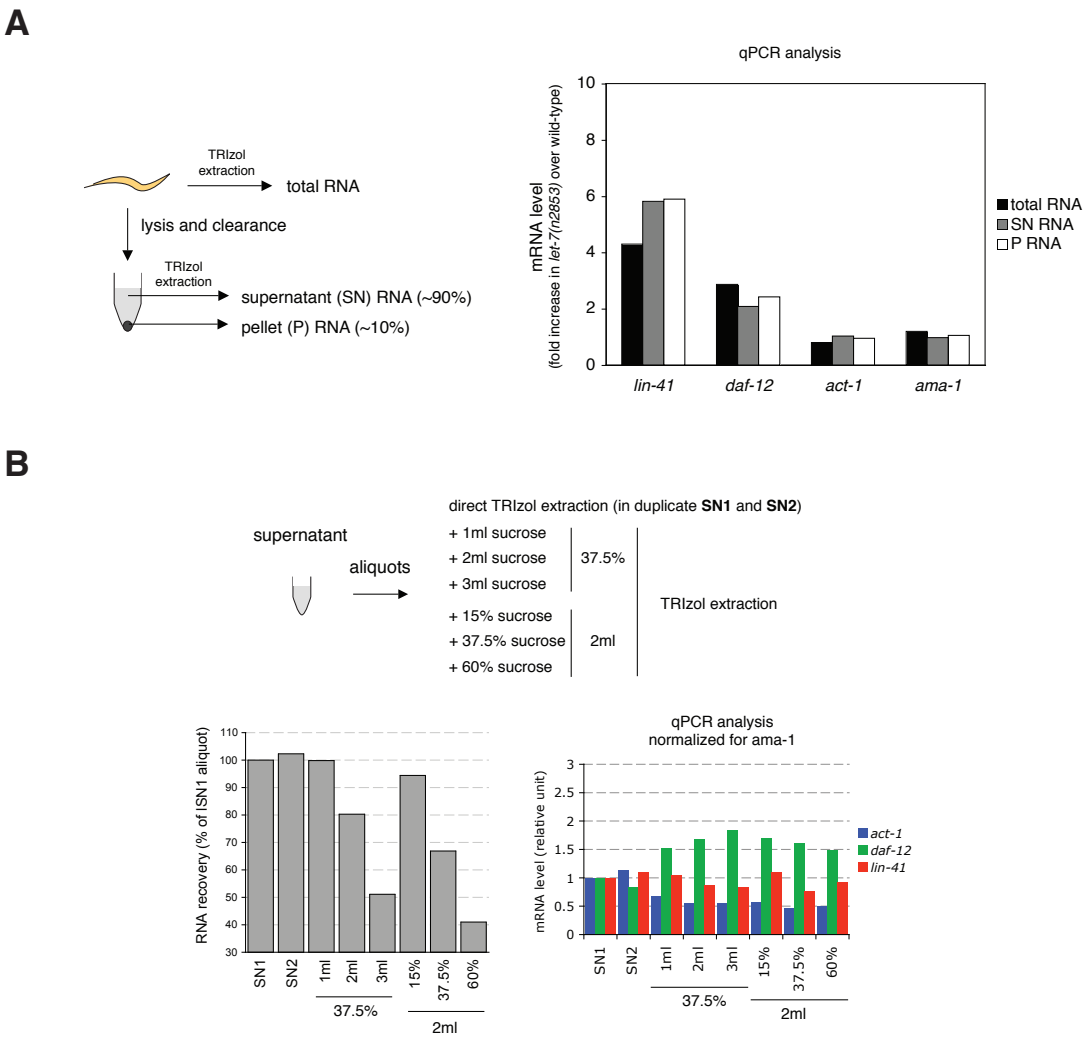

Figure S2: Analysis of RNA recovery from *C. elegans* lysate and polysome profile sucrose fractions.
(**A**) Synchronized L4 wild-type and *let-7(n2853)* animals were either resuspended in TRIzol or in polysome profile lysis buffer and crushed in mortar and pestle precooled with liquid nitrogen. RNA from worms resuspended in TRIzol was purified according to manufacturer's instruction. Lysate of worms resuspended in lysis buffer was cleared by centrifugation (see material and methods). RNA from the supernatant and the pellet was extracted using TRIzol. Approximately 90% of the RNA that can be recovered from direct TRIzol extraction is found in the supernatant for both wild-type and *let-7(n2853)* animals as determined by spectrophotometric analysis. Equal amounts of each RNA samples were reverse transcribed using random hexamer and relative abundance of the two *let-7* tragets *daf-12* and *lin-41* and of the two control genes *act-1* and *ama-1* was determined by qPCR. No enrichment of any mRNA can be detected in the supernatant or pellet samples as compared to total RNA. (**B**) Aliquots from one whole worm lysate (corresponding to SN in (A)) were either used for direct RNA extraction or mixed with sucrose solutions to obtain the indicated volume and concentration before RNA extraction. Although the amount of RNA recovered form lysates decreases when mixed with higer volume or higher concentration of sucrose solution, the relative abundance of *let-7* targets (*daf-12* and *lin-41*) and of *act-1* is only modestly (less than two-fold) affected as determined by random hexamer-primed RT-qPCR. Nevertheless, the sucrose concentration of polysome profile fractions was adjusted to 30% (w/v) before RNA purification in order to avoid underestimating the amount of RNA present in the deep part of the gradients.

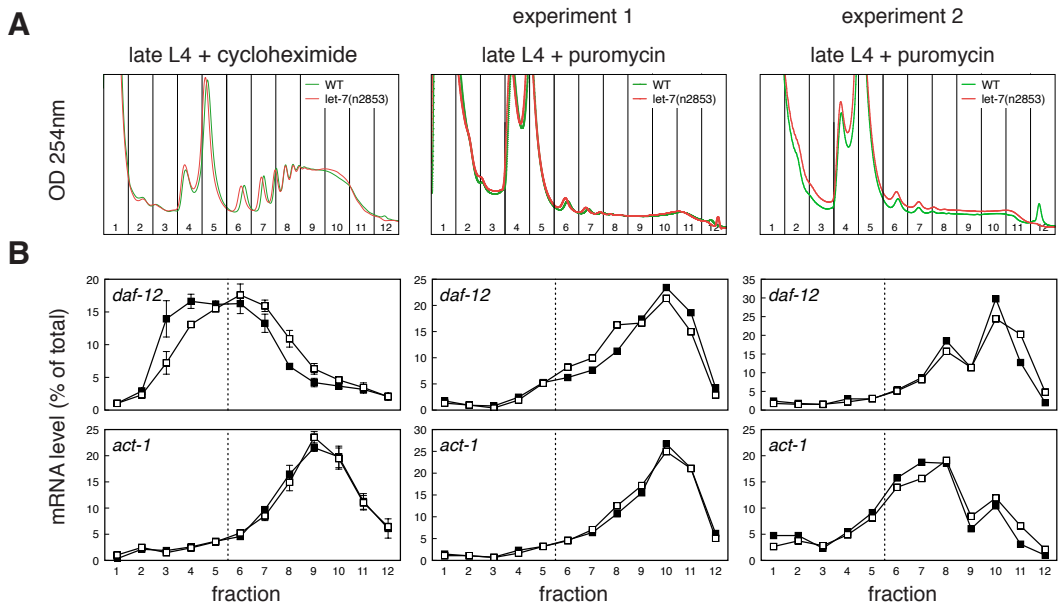

Figure S3: Puromycin treatment of *C. elegans* extract induces polysome dissociation.
(**A**) Typical polysome profiles from synchronized late L4 wild-type and *let-7(n2853)* animals in the presence of cycloheximide or puromycin. The solid line represents the separation between the (sub)monosomal and polysomal fractions. As indicated by absorbance profiles at 254 nm cycloheximide treatment preserves polysome integrity, whereas puromycin treatment induces polysome dissociation. Note that the absorbance profiles with cycloheximide are those also shown in Fig. 1 and S1. (**B**) Distribution of the *let-7* target *daf-12* and of the control gene *act-1* across polysome profiles from synchronized wild-type and *let-7(n2853)* late L4 animals treated with cycloheximide or puromycin. The dotted line represents the separation between the (sub)monosomal and polysomal fractions. Puromycin treatment collapses the elongation competent polysomes and no difference can be observed for *daf-12* distribution between wild-type and *let-7(n2853)* animals, indicating that the differential distribution observed in cycloheximide treated samples is due to differences in ribosome load on *daf-12* mRNAs. Surprisingly, all mRNAs, including mRNAs not targeted by miRNA, shift to denser fractions of the gradients under these conditions, suggesting that the ribosome-free mRNAs aggregate. For cycloheximide treatment, the averages of four biological replicates is shown and error bar are SEM, data are as in Fig. 1 and S1 and included for comparison.

Ding_FigS4

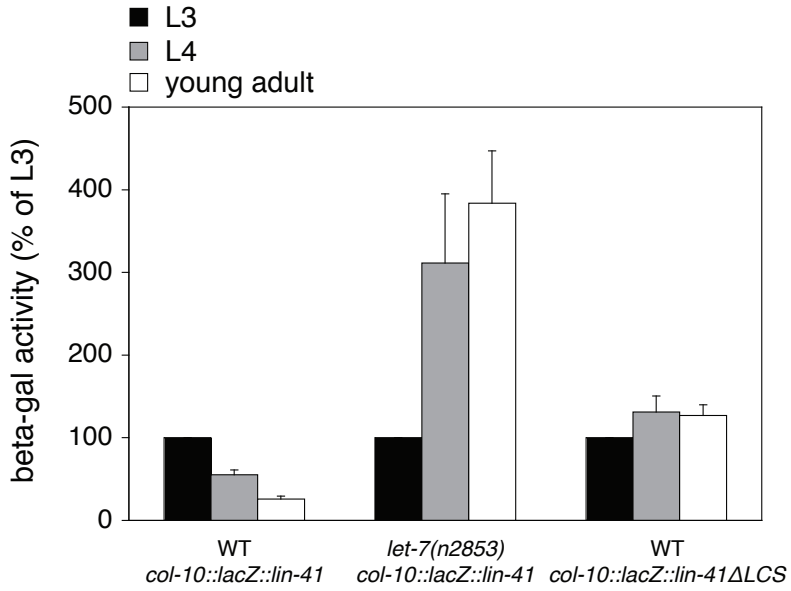

Figure S4: *lin-41* 3'UTR confers *let-7* mediated regulation
Quantitative measurement of the β-galactosidase (β-gal) activity in the different col-10::lacZ::lin-41 reporter strains shows that protein production is increased for the transgene lacking the *lin-41* LCSs or in *let-7* mutant worms, resulting in a respective five- and fifteen-fold increase in young adult compared to the activity in WT worms expressing a construct containing a full length *lin-41* 3'UTR. The lower level of β-Gal activity seen with the ΔLCS construct may indicate that other regulatory sites are still present in this 3'UTR. Averages of three biological replicates are shown, error bars are SEM.

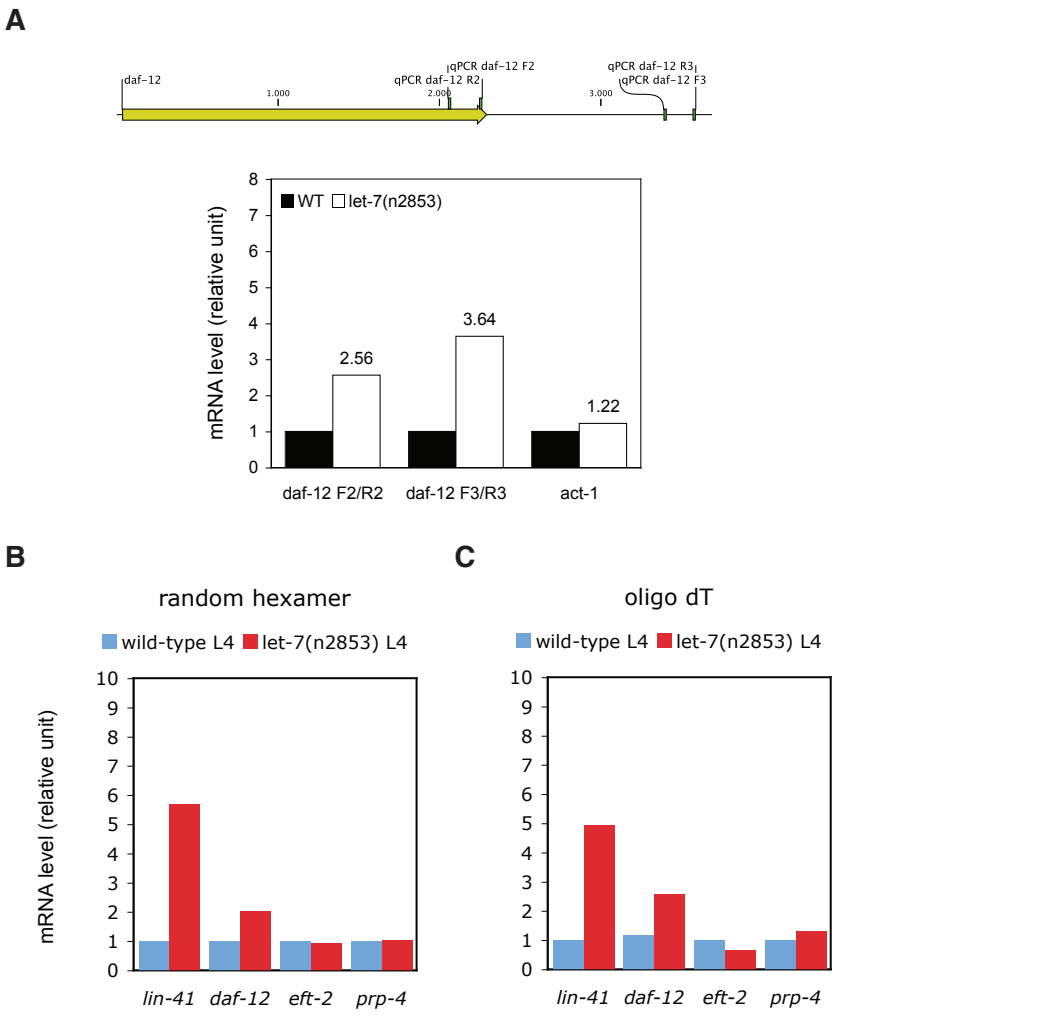

Figure S5 RT-qPCR validation
(**A**) *daf-12* total mRNA levels of synchronized late L4 wild-type and *let-7(n2853)* worms were determined by RT-qPCR using two distant pairs of primers: "qPCR daf-12 F2/R2" which amplify a fragment in the coding region and "qPCR daf-12 F3/R3" which amplify a fragment in the 3'UTR. The similarity of the results obtained with both primer pairs indicates that full length mRNA only is quantified.
(**B-C**) Reverse transcription reactions were performed on aliquots of the same total RNA extracted from wild-type and *let-7(n2853)* late L4 synchronized worms. The relative abundance, as determined by qPCR, of two *let-7* targets (*lin-41* and *daf-12*) and of two control genes (*eft-2* and *prp-4*) was found to be similar for reactions using random hexamer (**B**) and oligo dT primers (**C**). This indicates that mRNA levels determined using random hexamer represent full length mRNAs and that any effect miRNAs might have on polyA tail lengths of their targets does not prevent efficient oligo dT-primed reverse transcription.

Ding_FigS6

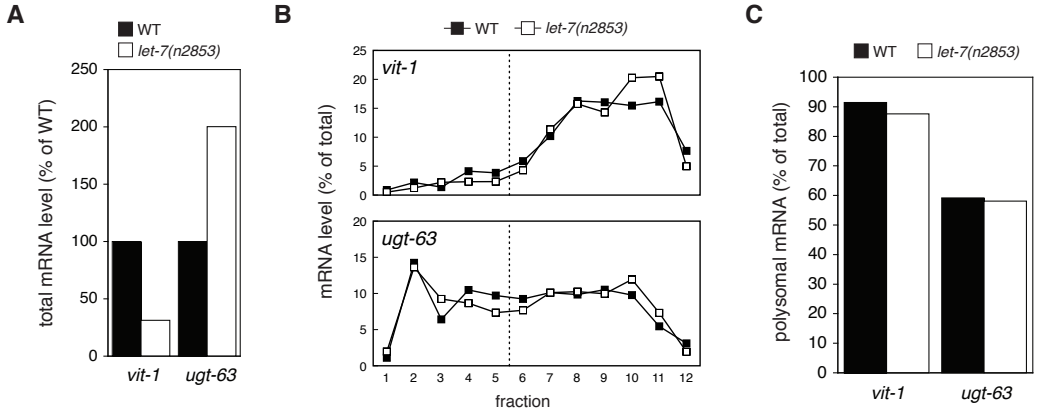

Figure S6: Changes in total mRNA levels do not influence translation initiation.
Although *vit-1* and *ugt-63* are differentially expressed in wild-type and *let-7(n2853)* animals, these genes are equally efficiently translated in both strains. (**A**) Analysis of *vit-1* and *ugt-63* total mRNA levels in synchronized late L4 wild-type and *let-7(n2853)* animals by RT-qPCR. (**B**) Distribution of *vit-1* and *ugt-63* mRNAs across polysome profiles from synchronized late L4 wild-type and *let-7(n2853)* animals. (**C**) Polysomal *vit-1* and *ugt-63* mRNA in late L4 wild-type and *let-7(n2853)* animals as percentage of the total. Averages of two biological replicates are shown for each panel**.**

Ding_FigS7

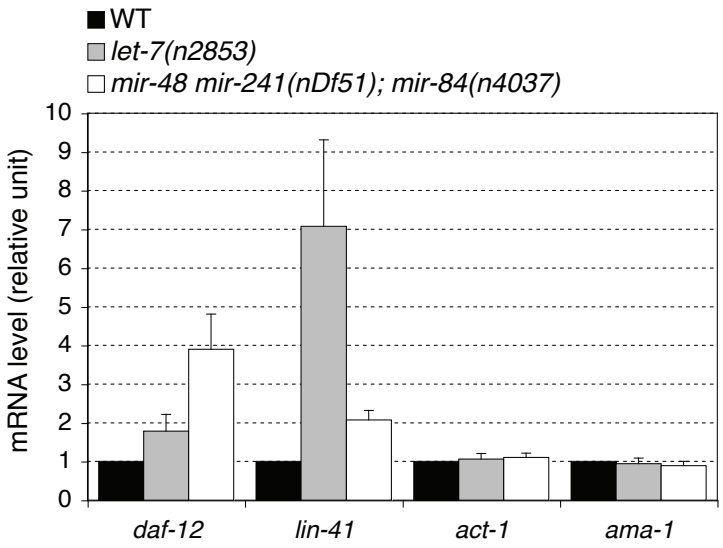

Fig. S7 Regulation of *let-7* targets by *let-7* sister miRNAs.
Analysis of the *let-7* target *daf-12* and *lin-41* and of control genes *act-1* and *ama-1* total mRNA levels in synchronized late L4 wild-type, *let-7(n2853)* and *miR-48 miR-241(nDf51); miR-84(n4037)* animals by RT-qPCR. Both *let-7* targets are also regulated by *miR-48*, *miR-84*, and *miR-241* although to different extents, which may be due to different temporal and spatial co-expression pattern with *daf-12* and *lin-41* (n=3, error bars are SEM).

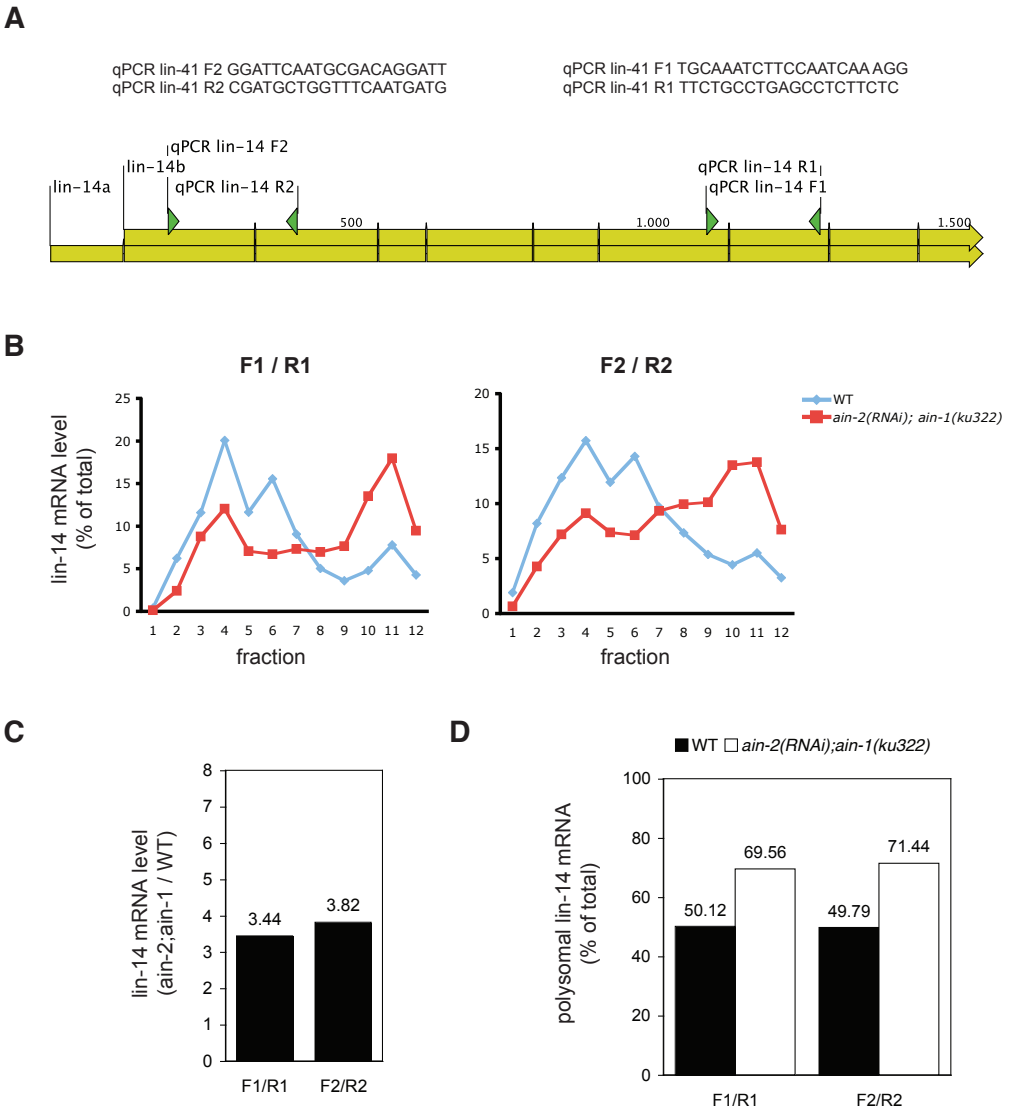

Figure S8 Comparison of two *lin-14* primer pairs
lin-14 total mRNA level and distribution across polysome profile of synchronized L4 wild-type and *ain-2(RNAi);ain-1(ku322)* animals were tested by RT-qPCR using two different pairs of primers amplifying distant regions from the same cDNA preparation. The similarity between the results obtained with both primer pairs indicates that full length mRNAs and not stabilized degradation products are quantified. (**A**) Schematic representation of the region amplified by RT-qPCR. (**B**) Polysome profile distribution. (**C**) Total mRNA level. (**D**) Percentage of mRNA in the polysomal fractions.

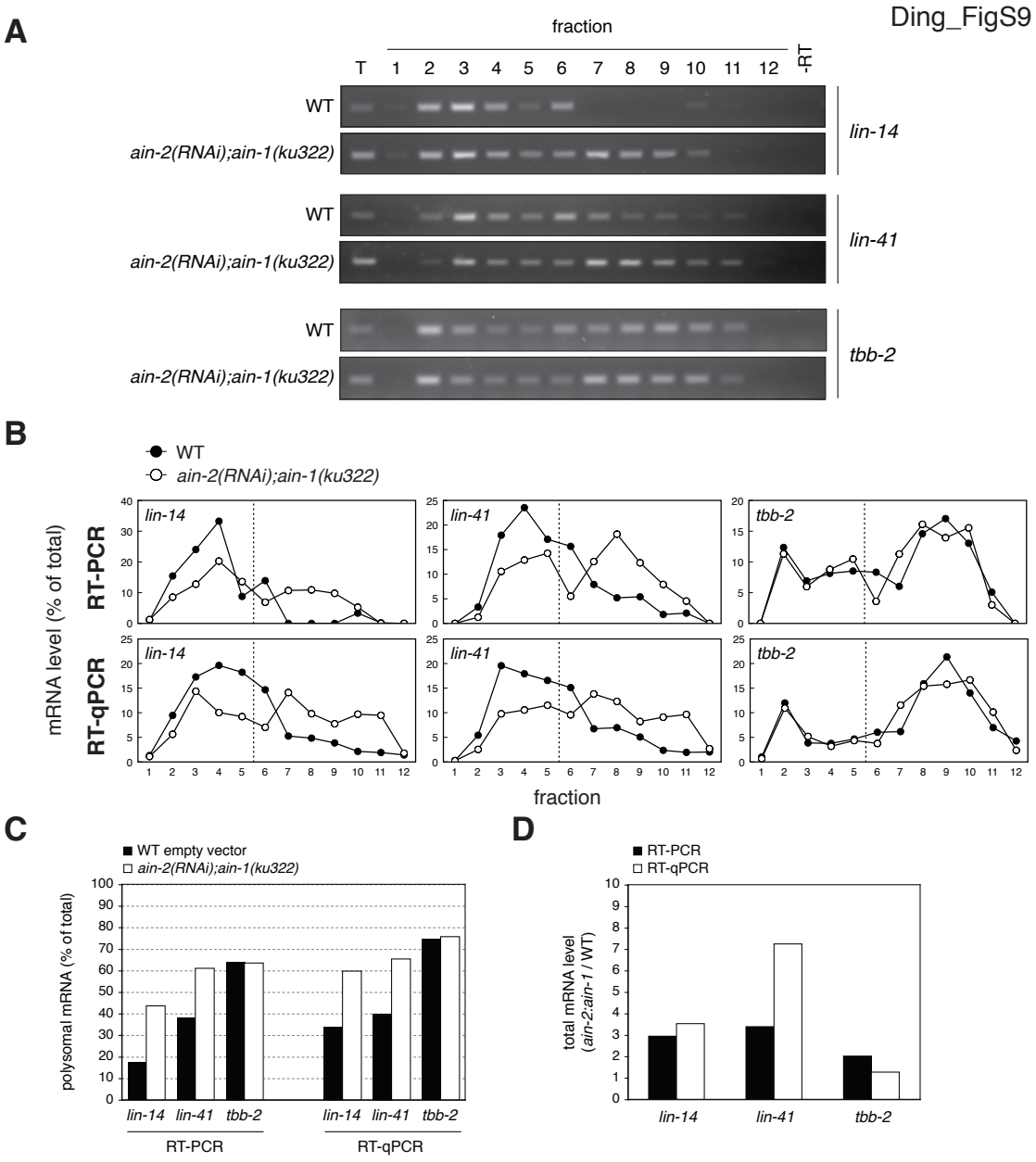

Fig. S9 Comparison between RT-qPCR and RT-PCR analyses.
(**A**) cDNA of total RNA and of RNA from polysome profile of synchronized L4 wild-type and *ain-2(RNAi);ain-1(ku322)* animals was used for semi-quantitative classical PCR. Aliquots of PCR reactions were analyzed on SybrSafe stained agarose gel. Distribution of both miRNA targets *lin-14* and *lin-41*, but not the *tbb-2* control mRNA, is shifted toward the polysomal fractions in *ain-2(RNAi);ain-1(ku322)* animals as compared to wild-type. Note that for each mRNA, wild-type and *ain-2(RNAi);ain-1(ku322)* samples were loaded on the same gel and photographed under identical conditions but are represented as separate pannels for better comparison. T, total RNA, -RT, negative control reaction lacking the reverse transcriptase using RNA from fraction 5. (**B**) Quantification of pictures shown in panel A was performed by densitometry analysis and is reported as percentage of the total. Note that values are corrected for total amount of RNA recovered from each fractions. Results from RT-qPCR analysis are shown for comparison. (**C**) Polysomal fraction of *lin-14*, *lin-41* and *tbb-2* mRNAs in synchronized L4 wild-type and *ain-2(RNAi);ain-1(ku322)* animals as percentage of the total determined by RT-PCR and RT-qPCR (**D**) Total levels of *lin-14*, *lin-41* and *tbb-2* mRNAs in synchronized L4 wild-type and *ain-2(RNAi);ain-1(ku322)* animals as fold increase in *ain-2(RNAi);ain-1(ku322)* animals compared to wild-type determined by RT-PCR and RT-qPCR. The overall results obtained by RT-PCR are similar to the ones obtained by RT-qPCR, confirming the validity of the latter method.

3.3. Additional results

3.3.1. Introduction

The results presented above show that *C. elegans* miRNAs repress target mRNAs by at least two mechanisms: mRNA degradation and inhibition of translation initiation, and that mutation of the GW182 homologs AIN-1 and AIN-2 is sufficient to prevent these two mechanisms. Moreover, the fact that *cog-1* repression by the *lys-6* miRNA seems to occur only at the level of translation suggests that mRNA degradation and translational control are two independent mechanisms, which often, but not always, occur concomitantly (Ding and Grosshans 2009).

Several questions remain to be addressed in order to understand in more detail how miRNAs function and what the exact interplay is between target translational repression and degradation. What are the exact molecular roles of AIN-1 and AIN-2? Do these factors physically repress target mRNAs or do they recruit additional effector proteins? What dictates the mechanism by which a target mRNA is repressed? The results presented in this section summarize preliminary experiments aimed at answering these points.

3.3.2. Results

AIN-1 specifically mediates miRNA target translational control

Because *C. elegans* miRNA targets are repressed by two different mechanisms, it is tempting to speculate that miRISC composition, which could differ in a tissue or time specific manner, might determine by which means a target is repressed. This idea is supported by the observation that, in *Drosophila* embryo lysates, Ago1- and Ago2-containing miRISCs employ different mechanisms to repress target reporters (Iwasaki et al. 2009). To test for a similar phenomenon in *C. elegans*, I analyzed the repression status of several miRNA targets in animals mutated for either *alg-1* or *alg-2*, the two *C. elegans* miRNA-specific Ago proteins, by polysome profile fractionation and RT-qPCR. Additionally, I also investigated the effects of individual *ain-1* or *ain-2* mutations on target repression. The results obtained were compared to the wild-type and *ain-2;ain-1* situations, in which full repression and, respectively, derepression occur.

In *alg-1(gk214)* or *alg-1(RNAi)* mutants, translational repression and mRNA degradation of *daf-12*, a *let*-7 target, and *lin-14*, a *lin-4* target, are both prevented to a similar extent as in *ain-2(RNAi);ain-1(ku322)* animals. Conversely, in *alg-2(ok304)* animals, *daf-12* and *lin-14* repression is not detectably impaired (data not shown). These results suggest that ALG-1 plays a major role in mediating miRNA function, whereas ALG-2 on its own does not seem to be essential. This observation is in line with the respective phenotypes associated with these mutants: *alg-1(gk214)* display strongly penetrant phenotypes (alae defects, retarded development timing, and vulva bursting), while *alg-2(ok304)* are superficially wild-type (Grishok et al. 2001), and suggests that the Argonaute composition of the miRISC does not directly determine the nature of the repression mechanism.

In *ain-1(ku322)* single mutant, polysome profile analysis revealed that translational repression of various *let-7* and *lin-4* targets is lost to a similar extent as in *ain-2(RNAi);ain-1 (ku322)* animals. On the other hand, translational repression of miRNA targets is as strong in *ain-2(tm1863)* as in wild-type animals (fig. 1A). Concerning target mRNA degradation, the total mRNA level of *daf-12* and *lin-14* in *ain-1(ku322)* is very close to the low level found in wild-type, while *lin-41* and *lin-28* levels are upregulated but not as much as in *ain-2;ain-1* double

mutants. Total mRNA levels of all these targets are unchanged in *ain-2(tm1863)* (fig. 1B) as well as in the likely null *ain-2(tm2424)* mutants (data not shown) when compared to wild-type. These results indicate that miRNA target translational repression is specifically mediated by AIN-1 and that AIN-2 on its own, similarly to ALG-2, does not seem to play an essential role in any repression mechanism. Importantly this also shows that, in *ain-1* mutants and for at least a subset of targets like *daf-12* and *lin-14*, mRNA degradation still occurs even when translational repression is lost. See figure 4A for a summary of these results.

Genetic interaction between AIN-1 and various RNA binding factors

In order to identify factors which, like AIN-1, might be involved in one specific mode of miRNA-mediated repression, I performed a small scale genetic screen on putative candidates. I hypothesized that depletion of miRNA effector proteins should produce a detectable phenotype in the sensitized *ain-1(ku322)* background, similar to *ain-2* depletion, which induces a phenotypic response in *ain-1* mutants but not in wild-type animals. I expected to identify factors which might be specific to the target mRNA degradation mechanism, like AIN-1 is to translational repression. Various factors were screened for their phenotypic effects when depleted in *ain-1(ku322)* and wild-type animals. The factors tested include RNA binding proteins known to interact physically with AIN-2 (*pab-2, sqd-1, car-1, tsn-1, aco-1*) (Zhang et al. 2007), and factors reported to interact with the miRISC (*tsn-1*, *vig-1*, *cgh-1, nhl-2*) (Caudy et al. 2003; Hammell et al. 2009). Qualitatively, depletion of only *pab-2*, *cgh-1*, and *sqd-1* showed and aggravation of *ain-1(ku322)* phenotypes. This was confirmed by quantitative analysis of alae defects and vulva bursting of wild-type and *ain-1* animals subjected to the respective RNAi (fig. 2). *pab-2*, *cgh-1*, and *sqd-1* depletions substantially increase alae defects in *ain-1(ku322)* animals but not, or only moderately, in wild-type (fig. 2A). However depletion of none of this factor reaches the effect induced by *ain-2(RNAi)* in *ain-1* mutants. Concerning the vulva bursting phenotype, it was only slightly increased by *pab-2*(*RNAi)* compared to the empty vector control, while in the case of *cgh-1* and *sqd-1* it equaled the effect of *ain-2* depletion. None of these RNAi conditions induced any bursting phenotype in wild-type animals (fig. 2B). These results indicate that *pab-2*, *cgh-1*, and *sqd-1* might be involved in the execution of miRNA mediated repression. However, because the effects of the depletion of these factors did not equal that of *ain-2*, it seems unlikely that they play an essential role in this pathway. A view

further supported by the observation that depletion of none of these factors in *ain-1* animals resulted in an upregulation of the *lin-4* target *lin-28*, as determined by western blot (data not shown).

A more direct way to discover potential mediators of miRNA action in general, and of target translational repression in particular, is to identify factors which physically interact with AIN-1. An antiserum, which specifically recognizes AIN-1 (fig. 3A and (Zhang et al. 2007)), was used for immunoprecipitation experiments. A specific ALG-1/2 antibody (fig. 3B) was used to validate AIN-1 co-immunoprecipitation. ALG-1 and ALG-2 are both strongly enriched in the wild-type immunoprecipitate, but absent from the *ain-1(ku322)* sample (fig. 3C). Of the genetically identified factors mentioned above, an antibody is available only for CGH-1, which was not found to co-precipitate with AIN-1 (fig. 3C). This experiment confirms that AIN-1 strongly interacts with ALG-1 and ALG-2 and indicates that, even though CGH-1 might play a role in the miRNA pathway, it is not tightly associated with miRISCs.

3.3.3. Figures

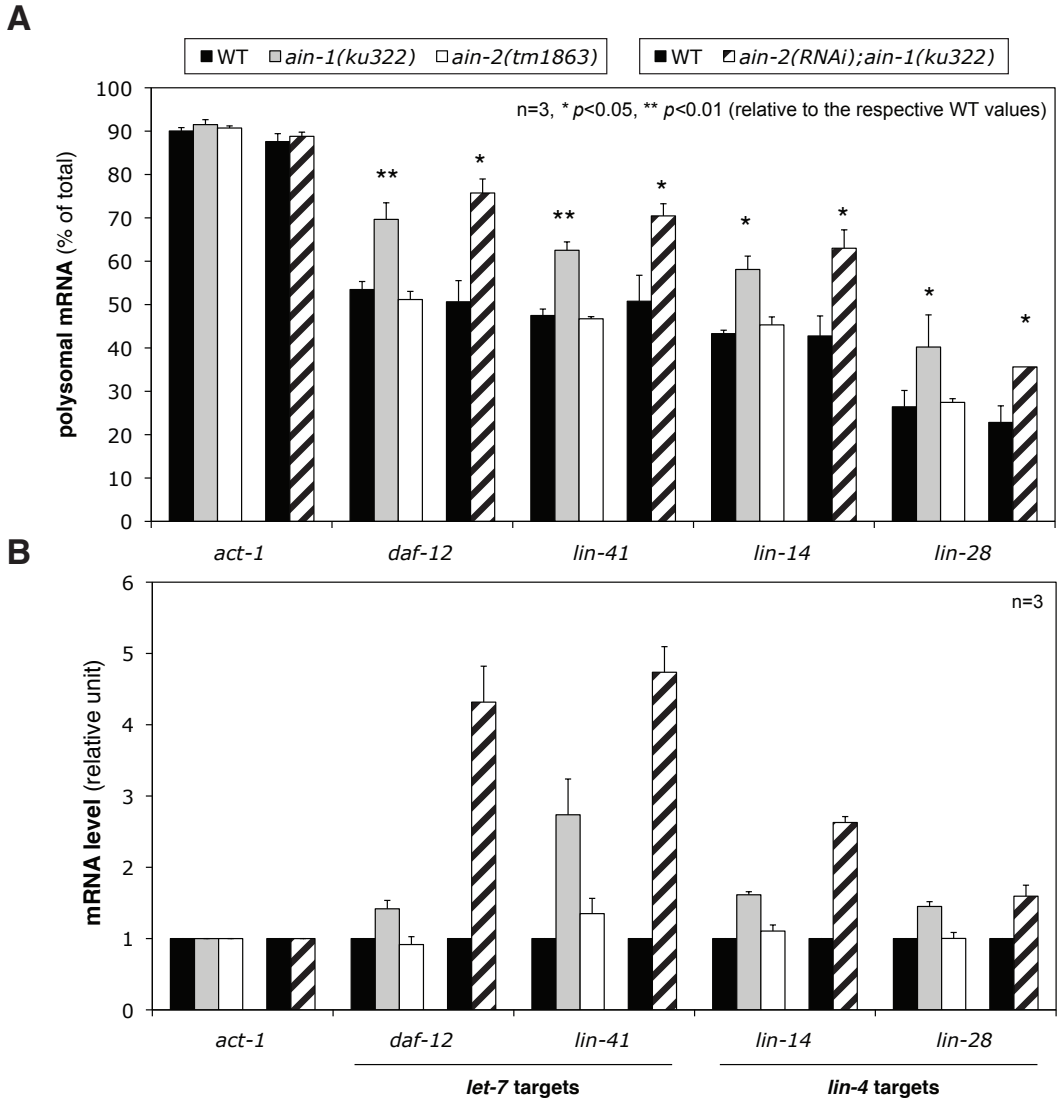

Figure 1 miRNA target translational repression is lost in *ain-1(ku322)* animals
Analysis of the translational status by polysome profile fractionation (**A**) and of the total mRNA level by qPCR (**B**) of the *let-7* targets *daf-12* and *lin-41,* the *lin-4* targets *lin-14* and *lin-28,* and of the control gene *act-1* in wild-type, *ain-1(ku322)*, and *ain-2(tm1863)* synchronized L4 animals. Results of *ain-2(RNAi);ain-1(ku322)* analysis (Ding and Grosshans 2009) are included for comparison.

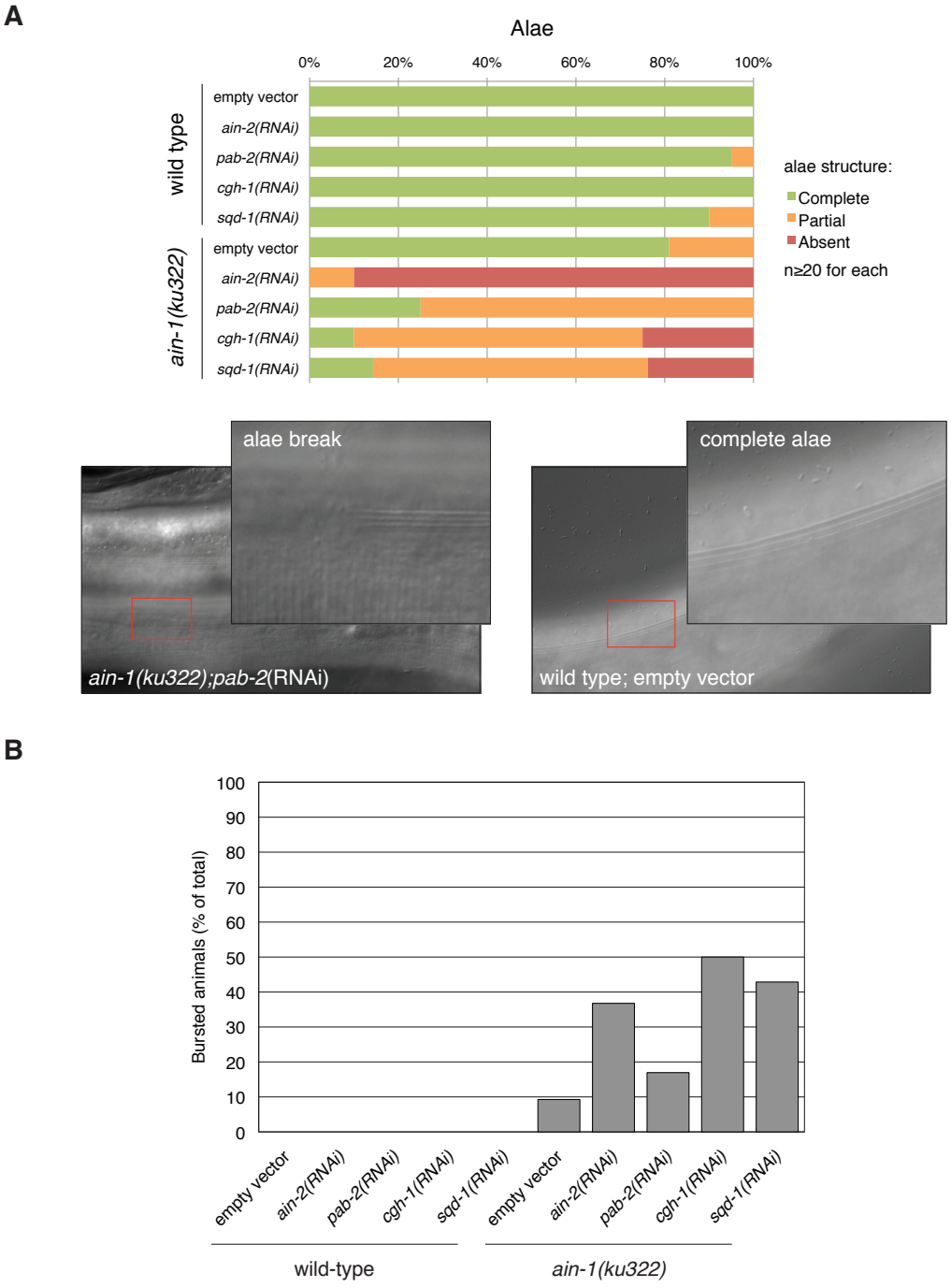

Figure 2 Genetic interaction between *ain-1* and various RNA interacting factors
(**A**) Alae structure analysis in wild-type and *ain-1(ku322)* young adult animals in response to depletion of *ain-2*, *pab-2*, *cgh-1*, and *sqd-1* by RNAi at 25 ºC. (**B**) Quantification of the vulva bursting phenotype in wild-type and *ain-1(ku322)* animals at the L4 to adult transition in response to depletion of *ain-2*, *pab-2*, *cgh-1*, and *sqd-1* by RNAi (n≥50 for each, as the *ain-1(ku322)* bursting phenotype is silenced at 20 ºC and above, this experiment was done at 15 ºC).

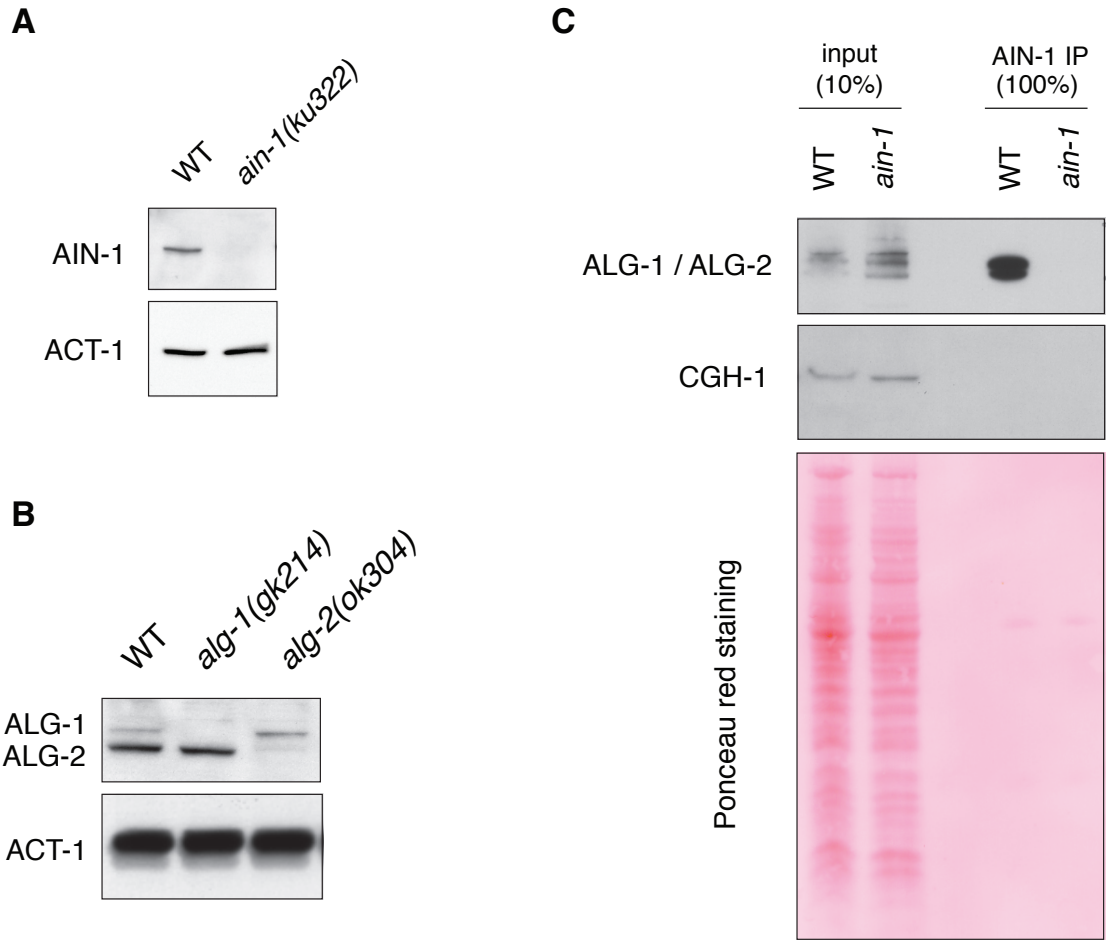

Figure 3 AIN-1 immunoprecipitation
(**A**) Western blot analysis of AIN-1 in wild-type and *ain-1(ku322)* animals. (**B**) Western blot analysis of ALG-1 and ALG-2 in wild-type, *alg-1(gk214)*, and *alg-2(ok304)* animals. (**C**) Immunoprecipitation of AIN-1 in wild-type and *ain-1(ku322)* animals. AIN-1 physically interacts with ALG-1 and ALG-2 but not with CGH-1.

A

	wild-type	*alg-1(gk214)* or *alg-1(RNAi)*	*alg-2(ok304)*	*ain-1(ku322)*	*ain-2(tm2424)* or *ain-2(tm1863)*	*ain-2(RNAi); ain-1(ku322)*
target mRNA degradation	✔	✘	✔	✔ (partially)	✔	✘
target translational repression	✔	✘	✔	✘	✔	✘
phenotypes* penetrance	null	strong	null	mild	null	strong

* alae defects, retarded development timing, vulva bursting

B

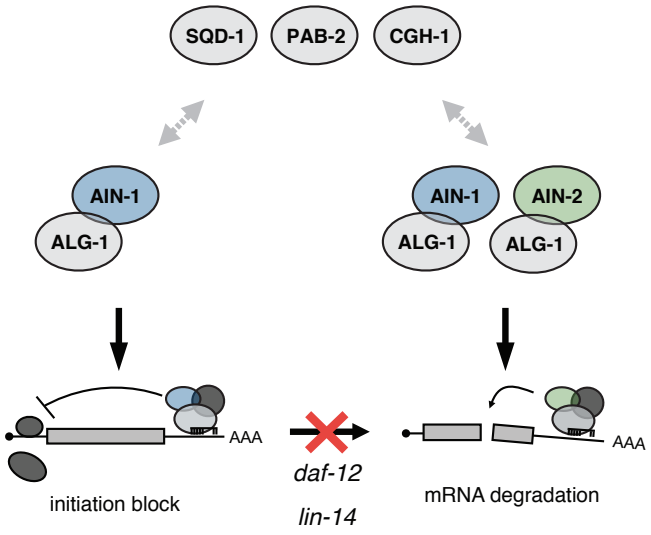

Figure 4 miRISC components role in miRNA mediated repression
(**A**) Effects of miRISC core factor mutations on specific repression mechanisms.
(**B**) Model of AIN-1 and AIN-2 role. Translational repression of miRNA targets is mediated by AIN-1, either directly or undirectly. Both AIN-1 and AIN-2 can apparently mediate miRNA target degradation. For some miRNA targets (*daf-12* and *lin-14*), mRNA degradation can still occur in the absence of translational repression suggesting that these two mechanisms might be independent pathways. Genetic data suggests that SQD-1, PAB-2, and CGH-1 might play an accessory role in the miRNA pathway. ALG-1, but apparently not ALG-2, is essential for both mechanisms.

3.3.4. Material and methods

C. elegans strains and RNAi

Wild-type N2, MH2385: *ain-1(ku322)*, VC446: *alg-1(gk214)*, and RB574: *alg-2(ok304)* were provided by the the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources, *ain-2(tm1863)* and *ain-2(tm2424)* by S. Mitani (National Bioresource Project, Japan), and *let-7(mg279)* (Reinhart et al. 2000) by F. Slack. RNAi was performed on synchronized population starting with L1 larvae and using a published RNAi feeding construct and protocol (Kamath et al. 2003; Grosshans et al. 2005).

Polysome profile analysis and RT-qPCR

Polysome profile and and total mRNA level analysis by RT-qPCR were performed using synchronized late L4 animals grown at 25 ºC as described in (Ding and Grosshans 2009).

Western blot and immunoprecipitation

Protein lysates were prepared by crushing worm pellet resuspended in one volume of lysis buffer (20mM HEPES, 10% Glycerol, 0.1% Triton X-100, 0.42 M NaCl, 1.5mM $MgCl_2$, 1 mM DTT, 1mM PMSF, complemented with EDTA-free protease inhibitor cocktail tablet (Roche Applied Science)) in mortar pre-cooled with liquid nitrogen. Lysates were cleared by centrifugation at 4 ºC for 10 min. at 10‘000 RCF. 30μg of protein samples were loaded on NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and transfered to Immun-Blot PVDF Membranes (Bio-Rad). AIN-1, ALG-1/2, CGH-1, and ACT-1 immunodetections were performed according standard protocols using AIN-1 antiserum (dilution 1/10’000, a gift from M. Han), anti-ALG-1/2 antibody (1/1’000 dilution, a gift from C. Mello), anti-CGH-1 antibody (1/2’000 dilution, a gift from K. Blackwell), and anti-Actin antibody (1/1‘000 dilution, MAB1501, Chemicon International).

Immunoprecipitation was done with the Immunoprecipitation Kit (Protein G) (11719386001, Roche Applied Science) according to the manufacturer’s recommendation. 700 μg of protein from *ain-1(ku322)* or wild-type lysates were used for immunoprecipitation with 2 μl of AIN-1 antiserum.

3.3.5. Discussion

Analysis of *alg-1* and *alg-2* mutants shows that these two factors do not have the same apparent importance in the execution of miRNA mediated repression. Both repression mechanisms are impaired in *alg-1* mutants, but none are in *alg-2* animals (fig. 4A). These two genes were thought to carry redundant functions because of their high homology (80% and 88% identical at the nucleotide and protein level, respectively (Grishok et al. 2001; Tops et al. 2006)) and the fact that they are synthetic lethal. However *in vivo* and *in vitro* data suggest that ALG-1 and ALG-2 perform non overlapping functions. For example there is no overlap in genes causing synthetic lethality in *alg-1* and *alg-2* respectively (Tops et al. 2006). ALG-1 and ALG-2 are ubiquitously expressed from early embryogenesis to adulthood and seem to associate with the same set of miRNAs, but to reside in complexes of different masses, as determined by size-fractionation (Tops et al. 2006). One possible explanation for ALG-1 and ALG-2 functional differences could therefore be that a mediator of miRNA function associate with ALG-1-, but not ALG-2-containing miRISCs. Indeed ALG-1 is found in a single complex larger than 650 kDa, whereas ALG-2 is present in two smaller complexes of about 250 and 500 kDa (Tops et al. 2006). Nevertheless, the identity of this hypothetical factor has yet to be determined. Although both ALG-1 and ALG-2 bind mature miRNAs and are core components of the miRISC, it was proposed that ALG-2 might function essentially by promoting pre-miRNA processing and facilitating the loading of mature miRNA on ALG-1, which would in turn mediate target repression (Tops et al. 2006). This model fits the data presented here. Target repression is lost in *alg-1* mutants, but, because pre-miRNA processing does not absolutely require ALG-2, miRNA mediated repression can still occur in *alg-2* mutants. Nevertheless, the fact that *alg-1(gk214)* is a null mutant (fig. 3B) and synthetic lethal with *alg-2* mutation indicates that these genes are likely to share additional partially overlapping functions.

Similarly to ALG-1 and ALG-2, AIN-1 and AIN-2 were postulated to have a redundant function (Zhang et al. 2007). This is based on partial homology between AIN-1 and AIN-2 and the observation that *ain-1* mutants display only weakly penetrant phenotypes, and that *ain-2* mutants look superficially wild-type, but that *ain-2;ain-1* double mutants show strong defects reminiscent of the phenotypes observed in worms deficient for miRNA function, such as delayed developmental timing, seam cells proliferation defects, and vulva bursting at the adult

transition (Zhang et al. 2007). For this reason, my initial experiments addressing the role of AIN proteins in the miRNA pathway were done using *ain-2(RNAi)*;*ain-1(ku322)* double mutant worms (Ding and Grosshans 2009). Here I show that individual loss of AIN-1 or AIN-2 does not have the same effect on miRNA target repression, indicating that these factors are not completely redundant. *ain*-1 mutation prevents target translational repression but not, or only moderately, target mRNA degradation. On the other hand, both repression mechanisms are fully functional in *ain-2* animals. These observations correlate well with the phenotype of the respective mutants: *ain-2(tm1863)* and *ain-2(tm2424)* are superficially wild-type and *ain-1 (ku322)* animals show mild defects in terms of alae structure, developmental timing, and bursting through the vulva (Fig. 2 and (Ding et al. 2005; Zhang et al. 2007)), which most likely result from loss of miRNA target translational repression. These phenotypes are more penetrant in *ain-2;ain-1* double mutants, possibly because both miRNA target repression mechanisms are prevented in this background (Ding and Grosshans 2009). The observation that the two mechanisms by which miRNA repress target genes can be uncoupled suggests that they represent distinct pathways (fig. 4B). If this conclusion could be further experimentally validated, it would answer a long standing question: miRNA target degradation does not require prior translational repression to occur. This possibility is supported by recent findings showing that, upon nutrient deprivation, *lin-14* mRNA degradation, but not protein downregulation, by *lin-4* is prevented, showing, here as well, that miRNA-mediated repression is enforced by pathways which can be uncoupled (Holtz and Pasquinelli 2009). Moreover, experiments in yeast showed that mRNA degradation can be initiated on actively translated mRNAs (Hu et al. 2009), further supporting the idea that target mRNA do not necessarily need to be translationally silent to be degraded.

Although, similarly to ALG-1 and ALG-2, AIN-1 and AIN-2 are core components of the miRNA pathway, it seems that these factors function exclusively downstream of the mature miRNA production, as no accumulation of pre-miRNA or decrease of mature miRNA can be observed in *ain-2;ain-1* double mutants (Zhang et al. 2007). The fact that target degradation is prevented only in *ain-2;ain-1* double mutants shows that expression of AIN-1 or AIN-2 is sufficient to mediate this function, or recruit a mediator thereof. On the other hand, AIN-1 is required miRNA target translational repression. It is possible that AIN-1 serves as a platform to recruit an inhibitor of translation initiation, which would not be able to bind AIN-2. It is also

possible that AIN-1 directly inhibits miRNA target translation. One way to test this possibility would be to attempt to rescue *ain-1(ku322)* animals using transgenes coding for various portions of AIN-1. If a minimal *ain-1* rescuing fragment can be identified, one could compare, by immunoprecipitation coupled to mass spectrometry, if the rescuing and non-rescuing fragments interact with the same set of factors. If this is the case, it would suggest that the concerned part of AIN-1 might be directly involved in translation repression, if not, this could reveal the identity of the factor mediating this activity.

There are several possible reasons to explain the functional differences between AIN-1 and AIN-2. First, as discussed above, they might recruit different downstream effectors, which, considering the relative low homology between AIN-1 and AIN-2 (Zhang et al. 2007), would not be so surprising. Second, they might be expressed in a different subset of tissues. Although a *gfp::ain-2* transgene was reported to be expressed ubiquitously (Zhang et al. 2007), the expression pattern of AIN-1 is not so clearly documented. L. Ding and colleagues, based on an *ain-1::gfp* translational reporter, reported that AIN-1 is expressed in "a variety of tissue types, including vulval precursor cells and multiple neurons" (Ding et al. 2005). Therefore it is not clear whether AIN-1 is ubiquitously expressed or not. Third, AIN-1 and AIN-2, as determined by *gfp* reporter transgenes, do not have the same subcellular localization: AIN-2 is diffuse in the cytoplasm (Zhang et al. 2007), whereas AIN-1 localizes in DCP-2-containing punctuate structures corresponding to P-bodies (Ding et al. 2005). However, it is not known if this difference in localization is a cause or a consequence of AIN-1 and AIN-2 functional differences. Additional experiments might help to tell apart these possibilities. For example, if a transgene expressing AIN-2 under the *ain-1* promoter can rescue *ain-1* mutants, this would indicate that AIN-1/2 functional differences are likely due to different expression patterns. Finally, it is also possible that the modality by which a miRNA target is repressed depends on its 3'UTR architecture, such as for example the presence of binding sites for additional regulatory factors. Therefore, additional miRNA targets should be analyzed to confirm that the observations made so far are specifically due to miRISC factor mutations and not the identity of the targets investigated.

Genetic data indicate that *pab-2, cgh-1,* and *sqd-1* might play a role in miRNA mediated repression. *pab-2* encodes the closest *C. elegans* homolog of the human cytoplasmic poly(A)-

binding protein (PABP) (Rhoads et al. 2006) and is likely to be involved in the general translation machinery. It is possible that the *ain-1* phenotype enhancement upon *pab-2* depletion results from general translation defects rather than a miRNA specific effect. The fact that *pab-2* RNAi did not enhance the weak vulva bursting phenotype of *let-7(mg279)* animals (data not shown), contrary to other factors involved in miRNA function (Parry et al. 2007), supports this idea. On the other hand, although *pab-2(RNAi)* failed to upregulate LIN-28 protein level in *ain-1*, it did so in wild-type animals (data not shown). This could be indicative of a partial overlap between AIN-1 and PAB-2 functions. Moreover, it was recently reported that, in mouse cell extract, GW182 interacts with PABP to promote miRNA target deadenylation (Fabian et al. 2009). A similar interaction could occur between AIN-1/2 and PAB-2, but this has still to be demonstrated.

CGH-1 is a conserved putative RNA helicase found in various RNA-protein granules, including P-bodies, and has been implicated in mRNA stability control and translational repression (reviewed in (Rajyaguru and Parker 2009)). This factor has recently been proposed to function together with the TRIM-NHL protein NHL-2 to modulate miRISC activity (Hammell et al. 2009). Enhancement of *ain-1* phenotypes upon depletion of *cgh-1* is in line with this idea, but the absence of effect upon *nhl-2* depletion is not. However this might be due to incomplete *nhl-2* depletion, as *nhl-2(ok818);ain-1(ku322)* animals have been reported to show enhanced alae defects compared to *ain-1(ku322)* (Hammell et al. 2009). Immunoprecipitation of CGH-1 coprecipitates miRISC components such ALG-1, ALG-2, and AIN-1 (Hammell et al. 2009), however the AIN-1 immunoprecipitation experiment presented here failed to pull down CGH-1. This could be due to the fact that CGH-1 interaction with AIN-1 is mainly RNA dependent or that this interaction is weak by nature. In any cases, NHL-2 and CGH-1 seem to play only accessory roles in the miRNA pathway, as none is absolutely required for miRNA mediated repression.

The third factor identified, SQD-1, is homologous to the Drosophila RNA binding protein Squid. *C. elegans* SQD-1 is poorly defined, but Squid has been shown to directly mediate sub-cellular localization of several mRNAs in Drosophila oocytes (Lall et al. 1999). It is therefore possible that SQD-1 might play a role in the relocalization of repressed miRNA targets, but

additional experiments are needed to substantiate this idea. Again, as far as partial depletion by RNAi allows to tell, *sqd-1* does not seem to be essential for miRNA function.

A comprehensive identification of factors physically interacting with AIN-1 and AIN-2 would help to better understand their molecular functions. Immunoprecipitation of endogenous AIN-1 works efficiently, as determined by the co-immunoprecipitation of ALG-1 and ALG-2. Coupled to mass spectrometry analysis, this approach would allow to determine if AIN-1 binds additional proteins or if it interacts only with DCR-1, ALG-1, and ALG-2, as published earlier (Ding et al. 2005; Zhang et al. 2007). A similar approach could be applied to AIN-2, either by using a tagged *ain*-2 transgene or by raising an AIN-2 specific antibody. However, for this factor, a list of 39 interacting proteins has already been published (Zhang et al. 2007). Therefore, it may be more fruitful to extend the small-scale genetic screen presented here to all these factors, by using *ain-1* or *alg-1* sensitized backgrounds and looking for specific enhancement of phenotypic defects. This would allow to determine if any of these factors is directly relevant to miRNA functions.

In summary, the additional data presented here show that *ain-1* and *ain-2* are not functionally redundant and that translational repression of miRNA targets is likely to be mechanistically independent of the degradation mechanism. As discussed above, further experiments are needed to firmly confirm these conclusions and understand in more details the functional role of AIN-1 and AIN-2. This is an important research aim, as it may offer us a more comprehensive view of miRNA biology, possibly relevant for new therapeutic approaches to miRNA related diseases (reviewed in (Brown and Naldini 2009)).

4. Conclusion

This study aimed at better understanding the *in vivo* mechanisms of miRNA-mediated repression. The use of *C. elegans* as a model organism allowed to circumvent the potential artifacts associated with *in vitro* or *ex vivo* systems and to study directly the action of endogenous miRNAs acting on endogenous targets in a physiologically relevant context. I postulated that that *C. elegans* miRNAs function *in vivo*, and at least partially, by inhibiting the initiation of translation on target mRNAs. The results presented here make this thesis the most plausible explanation for the biological phenomena observed, which is as close to the truth as one can be.

It is satisfying to achieve the validation of this thesis, as sanctioned by international peer-reviewed publications. It represents for me the successful management of a project on the long term and the resolution of numerous technical and intellectual challenges. It answers a few questions, but most importantly open new ones, concerning for example the modalities of the mechanism uncovered. I can only hope that this work will challenge or inspire others to attempt to answer them, which will hopefully in turn bring new interrogations, as questioning is the essence of scientific reasoning.

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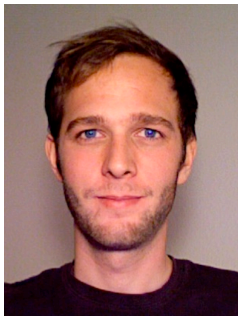
6. Curriculum vitae



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Publications

Hurschler B. A., Ding X. C. & Großhans H.
Translational Control of Endogenous MicroRNA Target Genes in *C. elegans*. ***Prog Mol Subcell Biol*** 50, 21-40 (2010)

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Protein Synthesis and Translationnal control, EMBL, Heidelberg.
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