Published in final edited form as:
Dev Biol. 2009 October 1; 334(1): 152–160. doi:10.1016/j.ydbio.2009.07.011.

Ribosomal protein RPS-14 modulates *let-7* microRNA function in *Caenorhabditis elegans*

Shih-Peng Chan and **Frank J. Slack**
Department of Molecular, Cellular and Developmental Biology, Yale University, P.O. Box 208103, New Haven, CT 06520

Abstract

The *let-7* microRNA (miRNA) regulates developmental timing at the larval-to-adult transition in *Caenorhabditis elegans*. Dysregulation of *let-7* results in irregular hypodermal and vulval development. Disrupted *let-7* function is also a feature of human lung cancer. However, little is known about the mechanism and co-factors of *let-7*. Here we demonstrate that ribosomal protein RPS-14 is able to modulate *let-7* function in *C. elegans*. The RPS-14 protein co-immunoprecipitated with the nematode Argonaute homolog, ALG-1. Reduction of *rps-14* gene expression by RNAi suppressed the aberrant vulva and hypodermis development phenotypes of *let-7(n2853)* mutant animals and the mis-regulation of a reporter bearing the *lin-41* 3′UTR, a well established *let-7* target. Our results indicate an interactive relationship between *let-7* miRNA function and ribosomal protein RPS-14 in regulation of terminal differentiation that may help in understanding the mechanism of translational control by miRNAs.

Keywords

microRNA; *let-7*; RPS-14; ribosome; stem cell; *C. elegans*

Introduction

MicroRNAs (miRNAs) are ~22-nucleotide small RNAs that regulate gene expression at the post-transcriptional level. In animals, miRNAs are involved in multiple biological functions, including development, apoptosis, metabolism and signaling pathways (Ambros, 2004; Bartel, 2004; Bushati and Cohen, 2007; Stefani and Slack, 2008). Dysregulation of miRNAs is also linked to human diseases and cancers (Bushati and Cohen, 2007; Esquela-Kerscher and Slack, 2006; Kloosterman and Plasterk, 2006). The first two known miRNAs, *lin-4* and *let-7*, were originally identified in the nematode *C. elegans* through genetic analysis and found to control the timing of cell fate determination during larval development (Lee et al., 1993; Reinhart et al., 2000). *lin-4* and *let-7* loss-of-function mutations disrupt the control of cell differentiation and cell cycle exit and result in inappropriate reiterations of the first larval stage (L1) and the fourth larval stage (L4) fates, respectively. For example, specialized hypodermal cells, known as seam cells, divide with a stem cell manner and terminally differentiate at the beginning of the adult stage in wild-type animals (Sulston and Horvitz, 1977). In *lin-4* and *let-7* mutant

To whom correspondence should be addressed frank.slack@yale.edu (FJS) Phone: 203-432-3492 Fax: 203-432-6161.

animals, seam cells fail to exit the cell cycle after the L4-to-adult transition and instead reiterate larval fates and continue to divide (Chalfie et al., 1981; Reinhart et al., 2000). Lack of cell cycle control and failure to terminally differentiate are hallmarks of cancer. Indeed, our previous studies have shown that *let-7* regulates the oncogene *RAS* in *C. elegans* and human lung cancer, which indicates a role for *let-7* as a tumor suppressor in carcinogenesis (Johnson et al., 2005).

In general, miRNAs, which are incorporated into ribonucleoprotein complexes (miRNPs) with Argonaute proteins and other effectors, regulate gene expression through base-pairing to target sites on the 3′ untranslated region (3′UTR) of mRNAs, which then reduces the protein yield. Several models of translation repression have been proposed from a variety of model systems, including *C. elegans, Drosophila* and mammalian cell cultures or *in vitro* translation in cell-free extract (see below). In these models, translational repression is a consequence of miRNP binding to mRNA sites with imperfect complementarity. The association of miRNPs with the translation machinery may trigger inhibition of translation initiation, inhibition of translation elongation, proteolysis of nascent peptides, pre-mature ribosome dissociation or mRNA degradation (Filipowicz et al., 2008; Nilsen, 2007; Pillai et al., 2007). However, there are points of controversy within these models. For example, studies in *C. elegans* and mammals have shown co-sedimentation of miRNAs with polysomes, which suggests miRNAs function at the elongation stage of translation (Maroney et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002). Recent studies in cell-free translation systems clearly promote a model where the binding of Argonaute proteins and GW182 to m^7G-cap interferes with translation initiation and may further induce deadenylation of the mRNA (Eulalio et al., 2008; Mathonnet et al., 2007; Standart and Jackson, 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007). Other studies also pointed out that miRNAs repress translation by preventing ribosome 60S subunit joining to mRNAs (Wang et al., 2008) and the *let-7* miRNA represses gene expression via translation initiation and elongation factors (Ding et al., 2008). On the other hand, little is known about the interaction between miRNP components and functional regions of the ribosome. In this study, we were interested in identifying factors that function with the *let-7* miRNA in developmental control.

Here we provide evidence in *C. elegans* that a ribosomal protein RPS-14 is involved in *let-7* miRNA function. The RPS-14 protein was detected in immunoprecipitation reactions with the nematode Argonaute protein ALG-1. We used RNAi to knock down *rps-14* expression and tested the effects on *let-7* miRNA function by examining vulval and hypodermal phenotypes of *let-7(n2853)* mutant animals (Reinhart et al., 2000). We also tested *let-7* regulation on a *lacZ* reporter gene that carries *lin-41* 3′UTR, which is a known *let-7* target (Reinhart et al., 2000; Vella et al., 2004). We found that loss of *rps-14* enhanced *let-7* function in *let-7 (n2853)* animals. These results demonstrate that RPS-14 protein, a component of the small subunit of the ribosome, negatively interacts with *let-7*.

Materials and Methods

Cell extract and immunoprecipitation

An expression construct, pBS-F48gfp, containing the *alg-1* genomic sequence and 4 kb of upstream promoter with recombinant GFP sequence fused to the 5′ end of the first exon was provided by Dr. Craig Mello (University of Massachusetts Medical School). This construct was injected into N2 wild type animals at a concentration of 100 ng/μl with *rol-6(su1006)* (Mello et al., 1991) as a coinjection marker at 70 ng/μl. Two representative lines carrying an extrachromosomal array were selected for integration using a UV-irradiation protocol: L4 worms were soaked in 30 μg/ml Trimethylpsolaren for 15 min and exposed to 365-nm light (30000 μJ/cm^2) in a Stratalinker UV Crosslinker 1800 (Stratagene). Integrants were screened from the progeny and outcrossed three times. Cell extracts were prepared as described

previously (Chan et al., 2008). Cell extracts were pre-cleared by incubation with 0.1X volume of protein-G Sepharose at 4°C for one hour. For western blotting, cell extracts containing 2 mg total protein were incubated with 20 μl of protein-G Sepharose beads, in which 2 μg of monoclonal anti-GFP antibody (clone 3E6, Invitrogen) were conjugated and crosslinked, at 4° C for one hour. The beads were washed four times with 1 ml washing buffer (50 mM Tris-HCl pH 7.5, 200 mM KCl and 0.05% NP-40). The proteins were extracted by boiling the beads in the SDS-PAGE loading buffer and separated on a 4-20% SDS-PAGE gel. For collecting distinct protein bands from the gels, cell extracts containing 10 mg total protein were used in the immunoprecipitation reaction with 10 μg of antibody. The beads were washed five times with high salt washing buffer (50 mM Tris-HCl pH 7.5, 500 mM KCl and 0.05% NP-40). The proteins were equally divided to two aliquots for two gels. One gel was stained by silver staining with a high sensitivity for detecting interesting bands. The other was stained by colloidal coomassive blue staining and was the actual gel used to cut the protein bands from. The proteins bands were analyzed by LC-MS/MS (ProtTech, Inc., PA).

For detecting miRNAs in the GFP::ALG-1 precipitates, cell extracts containing 1 mg total protein were used in the immnunoprecipitation reaction with 2 μg of antibody. The beads were treated with proteinase K (2 mg/ml proteinase K, 10 mM EDTA and 0.2% SDS) at 37 °C for one hour and the total RNAs were extracted by phenol/chloroform. *let-7* and *lin-4* miRNAs were detected by northern blot analysis using labeled DNA oligonucleotide probes as previously described (Esquela-Kerscher et al., 2005).

RNAi experiments

Gene knockdown was performed by feeding RNAi using previously published libraries and methods (Kamath et al., 2003; Timmons and Fire, 1998). The experiments were performed on NGM plates that contain 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and *E. coli* HT115 carrying pL4440-based dsRNA expression plasmids as a food source. Around one hundred synchronized *let-7(n2853)* L1 larvae were placed on the plate and then incubated at 20 °C. Five independent experiments were performed at 20°C for *rps-14* RNAi (total $n > 600$) and mock RNAi (total $n > 600$). The mock RNAi was performed using *E. coli* HT115 carrying the pL4440 vector without a targeting dsRNA sequence as the food source. After the L4-to-adult molt, viable animals were counted. Since *rps-14(RNAi)* has a moderate slow-growth effect, the intensity of *rps-14(RNAi)* was reduced by diluting the *rps-14(RNAi)* expressing bacteria with 1×, 2× or 4× volumes of bacteria carrying pL4440. These experiments were repeated 3 to 5 times with >100 animals counted in each single experiment. We also performed mock and *rps-14(RNAi)* with the animals carrying an integrated *wIs79[ajm-1::gfp; scm-1::gfp]* allele in the *let-7(n2853)* background ($n = 57$), to determine the effect of *rps-14(RNAi)* on seam cell development. The experiment was performed at 20°C and the number of seam cell nuclei and the adult alae were scored just after the L4-to-adult molt and before vulva bursting in a four-hour window. In this experiment, *rps-14(RNAi)* expressing bacteria was diluted in 4× volumes of bacteria carrying pL4440.

lacZ reporter gene expression

lacZ reporter expression under the control of the seam cell-specific *col-10* promoter and the regulation of *lin-41* 3′UTR was assayed as previously described (Reinhart et al., 2000; Vella et al., 2004). The construct pFS1030 contains the *col-10* promoter following the *lacZ* reporter gene and 1.1 kb sequence of *lin-41* 3′UTR (Vella et al., 2004). The control construct pFS1031 contains the *col-10* promoter following the *lacZ* reporter gene and *lin-41* 3′UTRΔLCS in which two major *let-7* binding sites are deleted. Both constructs were injected into N2 wild type animals at a concentration of 10 ng/μl with *rol-6(su1006)* as a coinjection marker at 80 ng/μl (Vella et al., 2004). The extrachromosomal DNA arrays were crossed to *let-7(n2853)* animals. Synchronized L1 larvae were subjected to RNAi experiments as described above but at 15 °

C. L3/L4 larvae and young adult worms grown from the same synchronized L1 larval population were harvested for X-Gal staining. Animals showing one or more stained seam cells were scored as positive. The relative percentages of adult animals with *lacZ* expression shown in Fig. 4 are obtained by dividing the percentage of stained adult animals by the percentage of stained larvae. Three independent experiments using *let-7(n2853)* animals carrying the *lacZ::lin-41* 3′UTR reporter were performed at 15°C (for *rps-14(RNAi)*, larva $n = 915$, adult $n = 223$; for mock RNAi, larva $n = 628$, adult $n = 359$ in total). An unpaired Student's *t*-test between two groups, i.e. mock RNAi and targeted gene RNAi, was used to determine whether the effect of the targeted gene RNAi was significant. Since *rps-14(RNAi)* has a slow-growth effect, the *rps-14(RNAi)* expressing bacteria were diluted in 2X volumes of bacteria carrying pL4440. For the control experiment using *let-7(n2853)* animals carrying the *lacZ::lin-41* 3′ UTRΔLCS reporter, larva $n = 249$, adult $n = 225$ for *rps-14(RNAi)*; larva $n = 243$, adult $n = 321$ for mock RNAi.

Gel shift assay

Synthetic *let-7* RNA (5′-UGAGGUAGUAGGUUGUAUAGU-3′) was purchased from Dharmacon Research Inc. and radiolabeled at the 5′ end using T4 polynucleotide kinase and [γ^{32}P] ATP. Reaction mixtures (25 μl) contained 1X kinasing buffer (NEB, 50 mM Tris pH 7.5, 5 mM $MgCl_2$), 2.0 μM RNA, 50 μCi of [γ^{32}P] ATP (3000 Ci/mmol, NEN) and 25 units of T4 polynucleotide kinase. The kinasing reaction was performed at 37 °C for 30 min. The specific activities of the RNAs were typically 1×10^6 c.p.m./pmol. The labeled RNA was purified by passage through a G25 column (Roche).

Gel shift reaction mixtures (10 μl) containing 20 mM Tris pH 7.5, 1 mM magnesium acetate, 1 mM calcium chloride, 0.01% NP-40, 2 mM ATP, 250 ng/μl *E. coli* tRNA, 4×10^5 c.p.m end labeled RNA (~40 nM) and cell extracts containing 20 μg total protein were incubated at room temperature for 60 min. The reaction mix was loaded into a 5% native polyacrylamide gel (19:1 acrylamide:bis) for electrophoresis. Gels were dried on 3 MM Whatman paper and exposed on a phosphoimager plate.

Results

In order to learn more about *let-7* miRNA mechanisms and function, we sought protein factors that interact with the *let-7* binding factor, Argonaute. We used the nematode Argonaute homolog ALG-1, known to bind to *C. elegans let-7* (Hutvágner et al., 2004), in immunoprecipitation reactions. We introduced a *gfp::alg-1* translational fusion construct into a *C. elegans* wild-type strain, N2. This *gfp::alg-1* construct was able to complement an *alg-1 (gk214)* knockout allele (Supplemental Figure 1), suggesting that the GFP::ALG-1 fusion protein is functional. We found that the GFP::ALG-1 protein is expressed throughout the animal, initiating expression in early embryos, and culminating in the adult with intense expression in the pharynx, seam cells, distal tip cells (DTCs), somatic gonad and vulval precursor cells (VPCs), vulva and spermatheca (Fig. 1). The GFP::ALG-1 protein is localized in the cytoplasm and can be detected highly concentrated in subcellular granules (Fig. 1N and 1P). The active expression of *alg-1* in the seam cells and vulva may reflect a requirement for miRNA-mediated regulation in the development of these cells and explain the heterochronic mutant phenotypes, including retarded seam cells and burst vulva in young adult animals, caused by RNAi knock down of Argonaute genes in *C. elegans* (Grishok et al., 2001). Cell extracts prepared from the transgenic line were used for immunoprecipitation with an anti-GFP antibody. We detected *let-7* and *lin-4* miRNAs in the precipitates, suggesting that GFP::ALG-1 is associated with, but not limited to, *let-7* miRNP (Fig. 2A). These results are consistent with previous reports implicating a function for ALG-1 in *let-7*-mediated heterochronic regulation (Grishok et al., 2001) and the presence of ALG-1 in a *let-7*-containing miRNP complex

(Hutvágner et al., 2004). We also detected *C. elegans* Dicer homolog DCR-1 and the ALG-1 interacting protein 1 (AIN-1) (Ding et al., 2005) in the precipitates (Fig. 2B), Dicer interacts with Argonaute proteins physically and incorporates into protein complexes processing miRNAs or siRNAs (Pham et al., 2004; Tahbaz et al., 2004). AIN-1 is the *C. elegans* homolog of GW182, a component of miRNA-induced Silencing Complex (miRISC), whose association with Argonaute proteins is required for miRNA-mediated translational repression (Ding et al., 2005; Eulalio et al., 2008). These observations indicate that GFP::ALG-1 incorporated into functional miRNP complexes. The precipitates from a large-scale immunoprecipitation reaction were subjected to SDS-PAGE followed by silver staining or colloidal coomassive blue staining. Twelve bands not present in the control lane were excised and sent for LC-MS/MS sequencing and fifteen proteins were identified by the mass spectrometry, including DCR-1 and ALG-1 (Fig. 2C and Supplemental Table 1). It is not clear if the ALG-1 peptides were from endogenous ALG-1 or degraded forms of the GFP::ALG-1 protein although the source bands migrated around the position of ALG-1 molecular weight (~110 kDa). In spite of that, the appearance of DCR-1 revealed that this procedure was able to detect relevant miRNP proteins.

Knocking down *rps-14* suppresses the vulval bursting phenotype of *let-7(n2853)* mutant animals

To test the relevance of any of these protein factors to *let-7*, we tested for a genetic interaction between ALG-1 interacting protein candidates and the *let-7(n2853)* mutant gene. *let-7 (n2853)* temperature sensitive mutant animals, in which the mutated *let-7* miRNA contains a G to A base substitution at the fifth nucleotide, die at the young adult stage by bursting through the vulva at the non-permissive temperature (20°C) (Fig. 3A) (Reinhart et al., 2000), while the phenotype is less penetrant at a lower temperature (15 °C). The weakly penetrant vulval bursting phenotype at 15°C can be enhanced by reducing expression of components in the miRNA pathway, ALG-1 or DCR-1, using RNAi (data not shown). Thus, the severity of the temperature sensitive *let-7(n2853)* phenotype can be altered by changing the activity of miRNP components. We used a feeding RNAi library (Kamath et al., 2003) to screen preliminary ALG-1 interacting protein LC-MS/MS candidates for their influence on viability of *let-7 (n2853)* mutant animals (Supplemental Table 1). We anticipated that some candidates may act as positive regulators, e.g. if these were functional effectors of *let-7* miRNPs, then knocking down them should cause enhancement of the bursting phenotype at the permissive temperature. In contrast, other candidates may act as negative regulators of *let-7* miRNPs and knocking down these factors may cause reduced bursting at the non-permissive temperature.

One candidate gene that interacted in this secondary screen encodes ribosomal protein RPS-14 (Supplemental Table 1). Knocking down *rps-14* by RNAi suppressed the vulval bursting phenotype of the *let-7(n2853)* mutant at the non-permissive temperature (88% survival, $n > 600$) (Fig 3B). *rps-14(RNAi)* also results in slow growth and sterility. We serially diluted the bacterial food used for feeding *rps-14(RNAi)* by adding 1X, 2X or 4X volumes of bacteria carrying a control plasmid pL4440, which was used for mock RNAi, to provide a lower intensity of RNAi. We found that the *let-7(n2853)* animals grew at a near normal rate at a 1:4 mix ratio and the bursting phenotype was still significantly suppressed (Fig. 3B). *rps-14 (RNAi)* strongly suppressed the vulval bursting phenotype of *let-7(n2853)* mutant animals, showing that reduced expression of *rps-14* partially relieves this particular defect in *let-7* loss-of-function animals. These results suggest a negative role of RPS-14 in *let-7* function.

Since knock down of another ribosomal small subunit protein RPS-24.2 also has been shown to suppress the vulval bursting phenotype of *let-7(n2853)* (Ding et al., 2008), we were interested in the effects of knocking down other ribosomal proteins even we did not detect them in our ALG-1 immunoprecipitation. We tested RNAi of 37 other ribosomal protein encoding genes

and found that *rps-25(RNAi)* and *rpl-24.2(RNAi)* (Ding et al., 2008), in addition to *rps-14 (RNAi)*, showed suppression of *let-7* vulva bursting whereas *rpl-29(RNAi)* did not (Supplemental Table 2). Knock down of the remaining ribosomal protein genes caused highly penetrant developmental arrest at early or late larval stages (Supplemental Table 2). Hence, the possibility that general ribosome deficiency affects the bursting phenotypes can not be completely excluded.

Knocking down *rps-14* suppresses *let-7(n2853)* mutant phenotypes in seam cells

rps-14(RNAi) strongly suppressed the vulval bursting phenotype of *let-7(n2853)* mutant animals at 20°C, showing that reduced expression of *RPS-14* partially relieves this particular defect in *let-7* loss-of-function animals. We tested if *rps-14(RNAi)* suppressed abnormal seam cell development in *let-7(n2853)* animals. In wild-type animals, the ten seam cells on each side of the body present at hatching become 16 seam cells during the L2 stage. At the third and fourth larval transitions, seam cells divide but only one daughter cell of each division retains the seam cell fate and the total number of seam cells remains the same. At the L4-to-adult molt, neighboring seam cells exit the cell cycle, fuse with each other and secrete a cuticular structure known as adult alae (Sulston and Horvitz, 1977). In *let-7(n2853)* animals, seam cells reiterate the late larval fate of cell division at the L4-to-adult molt, which leads to an increased number of seam cell nuclei, around 22~24 (Reinhart et al., 2000). Also, those seam cells do not fuse with neighboring seam cells and do not generate alae. We scored the number of seam cell nuclei, using seam cell marker (*scm-1::gfp*) expression from the *wIs79[ajm-1::gfp; scm-1::gfp]* allele, and scored the formation of alae in *let-7(n2853)* mutants just after the L4-to-adult molt under mock or *rps-14(RNAi)* (with a reduced RNAi intensity by adding 4X volumes of bacteria carrying the pL4440 plasmid, see Material and Methods). In our experiments, *let-7(n2853)* animals after the L4-to-adult molt displayed 22.3 ± 1.6 seam cell nuclei (n = 110) on average. *rps-14(RNAi)* partially suppressed the abnormal reiterative division of seam cells and reduced the number to 17.9 ± 0.5 (n = 57) (Fig. 3C). *rps-14 (RNAi)* also significantly restored alae formation in *let-7(n2853)* worms after the L4-to-adult molt; 80% of animals produced complete alae (Fig. 3D and 3E) upon *rps-14(RNAi)*, compared to 16% in the mock RNAi experiment (Fig. 3E). These results suggest a genetic interaction between *rps-14(RNAi)* and *let-7*, and reinforce the notion that RPS-14 interacts negatively with *let-7*.

Knocking down *rps-14* enhanced *let-7*-mediated gene regulation in *let-7(n2853)* animals

It is possible that the suppression on the *let-7(n2853)* mutant phenotypes in our experiments was due to *rps-14(RNAi)* solely affecting genes downstream to *let-7* in the *C. elegans* heterochronic gene pathway (Reinhart et al., 2000) and not because it interfaced with *let-7* function. To distinguish between these possibilities, we tested if direct *let-7* regulation on a reporter with *lin-41* 3′UTR would be affected by *rps-14(RNAi)*. *let-7* is up-regulated from the third larval (L3) stage during *C. elegans* development and binds to the 3′UTR of the *lin-41* gene to repress its expression in seam cells. This event allows seam cell fates to transition normally from the larval to adult stage. The 1.1 kb of sequence after *C. elegans lin-41* stop codon, which contains *let-7* binding sites, was fused to a *lacZ* reporter gene driven by the *col-10* promoter (Reinhart et al., 2000). The β-galactosidase activity, which is specifically expressed in seam cells, can be detected by X-Gal staining (Wightman et al., 1993). This construct was introduced into N2 and *let-7(n2853)* animals. In N2 animals, robust X-Gal staining was observed in the seam cells through the L1 to the L4 larval stages but the staining was greatly reduced at the adult stage (46% of animals, data not shown) (Reinhart et al., 2000; Vella et al., 2004). In contrast, the X-Gal staining in *let-7(n2853)* adult animals was unchanged from larval levels (98%, n = 359, normalized to stained larvae, see Materials and Methods), indicating a low efficacy of *let-7* regulation (Fig. 4A and 4C). Upon *rps-14 (RNAi)* (with a reduced RNAi intensity by adding 2X volumes of bacteria carrying the pL4440

plasmid, see Material and Methods), only 56% of *let-7(n2853)* adult animals were stained (n = 223, Student's t-test p = 0.011), suggesting that *let-7* function in *let-7(n2853)* mutants was partially restored. We also used a reporter construct with the *lin-41* 3′UTRΔLCS as control, in which the two major *let-7* binding sites in the 3′UTR have been removed (Vella et al., 2004). In the control experiment, we found little difference in the staining in larvae and adult animals regardless of whether mock or *rps-14* RNAi was performed (Fig. 4D), which suggested the effect of *rps-14(RNAi)* is not due to defects in general ribosome function, but is specific to *let-7*. These results support our assertion that RPS-14 acts as a negative modulator of *let-7* function.

rps-14(RNAi) affects *let-7* miRNP formation

We previously reported that *in vitro* the *let-7* miRNA assembles into ribonucleoprotein complexes of different sizes, named M* (>667 kDa), M3/M4 (~500 kDa), M2 (~250 kDa), M1 (~160 kDa) and M** (Chan et al., 2008). The protein content and the role of these complexes are still not clear. Two small complexes M1 and M2 assemble only with the members of the *let-7* miRNA family and their formation is highly dependent on specific sequences in the 5' seed region of *let-7*. The formation of M1 and M2, especially M1, also requires ALG-1 (Chan et al., 2008), suggesting its relevance to miRISCs. A smaller complex M** migrates faster than M1 on native gels as a less distinct band and may represent heterogeneous initial products in assembly or disassembly (Chan et al., 2008). We incubated radioactive labeled *let-7* with the cell extract prepared from animals subjected to *rps-14 (RNAi)* (with a reduced RNAi intensity by adding 1X volume of bacteria carrying the pL4440 plasmid, see Material and Methods) and tested for *let-7* complex formation. The amount of M** was reduced and the formation of M1 was slightly increased (Fig. 5). While we found an increase in the M1/M** ratio, other complexes seemed not to be affected significantly. Although the *let-7* binding complexes are still to be characterized in detail, this result shows that RPS-14 is required for these complexes to form and suggests that RPS-14 modulates *let-7* function through miRNP complex formation (see Discussion).

Discussion

We found a small ribosomal protein RPS14 as an ALG-1 co-purifying product. In our experiments, *rps-14(RNAi)* significantly suppressed the vulval bursting phenotype of *let-7 (n2853)*. *rps-14(RNAi)* also showed a strong genetic interaction with *let-7* in a particular developmental event in seam stem cells. Moreover, *rps-14(RNAi)* restored *let-7* mediated regulation of the *lacZ* reporter gene carrying *lin-41* 3′UTR in *let-7(n2853)* animals. Although knocking down *rps-14* also caused slow growth, perhaps because translation is impaired, the expression of a *lacZ::lin-41* 3′ UTR reporter gene at all larval stages was not affected, and neither was the expression of a *lacZ::lin-41* 3′UTRΔLCS reporter gene affected at larval or adult stages. Thus, the effect of *rps-14(RNAi)* on enhancing *let-7* function seems to be specific. In addition, *rps-14(RNAi)* altered the formation of certain *let-7* binding complexes in cell extracts. These *in vivo* and *in vitro* results consistently suggest a negative impact of RPS-14 on *let-7* miRNA function.

Since in human cells, *let-7a*, Ago proteins and reporter mRNA carrying the *lin-41* 3′UTR have been shown to sediment with polyribosomes in sucrose gradients (Nottrott et al., 2006), we suspected that our immunoprecipitates may contain certain ribosomal components. However, we did not find any other ribosomal proteins in our band samples. Nevertheless, we tested whether knock down of other ribosomal proteins could suppress the vulval bursting phenotypes of *let-7(n2853)*, and found that like *rps-14(RNAi)*, *rps-25(RNAi)* and *rps-24.2(RNAi)* also showed similar effects whereas *rpl-29(RNAi)* did not. Hence, it is difficult to rule out whether the slowed growth caused by knocking down ribosomal proteins contributed to the suppression

on vulva bursting of *let-7(n2853)* adult animals. In spite of that, our findings still strongly suggest a negative role of RPS-14 in *let-7* function.

It is interesting that *rps-14(RNAi)* altered formation of *let-7*-binding complexes *in vitro*. Although the roles of these complexes in *let-7* function are still unclear, *rps-14(RNAi)* seems to facilitate the formation of at least one complex (M1) and prevent another *let-7*-binding product (M**). In a pulse-chase experiment in our previous report, we found that the ^{32}P-labeled *let-7* in these small complexes, including M2 and M1, can be rapidly replaced by a cold *let-7* competitor (Chan et al., 2008), not only suggesting that the *let-7* miRNA in these complexes is highly exchangeable but also indicating that the pre-formed complexes with endogenous *let-7 in vivo* can be monitored by adding exogenous ^{32}P-labeled *let-7* into cell extracts. In other words, the signals in our gel shift experiment may represent both endogenous *let-7* complexes and newly *in vitro* assembled complexes. This is also supported by the observation of less M1 and M2 complex formation in the *alg-1(gk214)* cell extract (Chan et al., 2008), in which the amount of endogenous *let-7*, probably also the amount of endogenous *let-7* binding complexes, is lower than that in the wild-type cell extract (unpublished data). However, the amount of M** in the *alg-1(gk214)* cell extract is higher than that in the wild-type cell extract, resulting in an very low M1/M** ratio (Chan et al., 2008). We speculate that deficiency in Argonaute proteins may promote *let-7* into the M** pool, where *let-7* is only associated with a few proteins. In contrast, *rps-14(RNAi)* reduced the amount of M** and increased M1/M** ratio, suggesting that the lack of RPS-14 affects *let-7* and/or miRISCs behavior and causes less *let-7* in the M** pool. Although the mechanism is unknown, this observation is consistent to the notion that RPS-14 plays a negative role in *let-7* function. It would be interesting to see if RPS-14 also affects other miRNAs. However, these small ribonucleoprotein complexes were found only with *let-7* and their formation is dependent on the 5' seed region sequence of *let-7* (Chan et al., 2008).

RPS-14 associates with helix 23 of the 18S rRNA during the processing of rRNA and following ribosome assembly. In other systems, it has also been found to be capable of auto-regulation of its own expression, transcriptionally or post-transcriptionally (Fewell and Woolford, 1999; Tasheva and Roufa, 1995). In human cells, two stable antisense RNAs transcribed from the first intron of *RPS14* gene stimulate *RPS14* mRNA transcription. Free RPS14 protein was able to bind *RPS14* mRNA and these antisense RNAs. It also inhibits its own mRNA's transcription directly by its negative effects on the *RPS14* mRNA transcription complex or indirectly by inhibiting the synthesis of stimulating antisense RNAs (Tasheva and Roufa, 1995). In yeast, free RPS-14 protein can inhibit *RPS-14B* expression by associating with sequences around the 5′ splice site of *RPS-14B* pre-mRNA, which form a stem-loop structure with a bulged region caused by imperfect complementary base pairs (Fewell and Woolford, 1999). These examples indicate that certain ribosomal proteins can be multi-functional and are able to target other RNAs besides rRNAs. However, our results do not yet show whether the free RPS-14 or the RPS-14 protein on the 40S ribosomal subunit is involved in *let-7* function. One hypothesis is that RPS-14 assembled on the 40S ribosomal subunit may play a role when ribosome activity is regulated by the *let-7* miRNA. Since the co-purification of RPS-14 with ALG-1 in our experiments could be due to the proximity of miRISC and ribosome, an interesting speculation is that RPS-14, not only associates with 18S rRNA, but may also interact with the approaching *let-7* miRNPs and hence play a role in translational control. In the 40S subunit, RPS-14 is on the “platform” and helps to form the mRNA exit channel along with its interacting protein RPS-5 in the “head” of the 40S subunit (Yusupov et al., 2001). It has been shown in bacteria that mutations in this region can reduce the association of ribosomal subunits (Robert and Brakier-Gingras, 2003). Taken together, we speculate that miRISC may prevent the 60S subunit from joining to the pre-initiation complex or disrupt translating ribosomes by interacting with ribosome 40S subunit surfaces near the RPS-14/RPS-5 region.

Some ribosomal proteins have been implicated in mi/siRNA silencing. For example, RpL5 and RpL11 were found in a complex containing dFMR, AGO2, Dmp68 and 5S rRNA in *Drosophila* (Ishizuka et al., 2002). The authors also showed that dFMR associates with Dicer and miRNAs *in vivo*. dFMR may associate with the 60S ribosomal subunit, by binding to RpL5 and RpL11 that are on the top surface of 60S ribosomal subunit, and prevent the assembly of the translation initiation complex (Ishizuka et al., 2002). In addition, a recent study in *Drosophila* has shown that knockdown of several ribosomal proteins impacted small RNA pathways. Among these ribosomal proteins, RpS7, RpL21 and RpL22 could immunoprecipitate mi/siRNAs and components of the miRNA or siRNA pathways, including AGO1, AGO2, dFMR and VIG (Vasa intronic gene) (Zhou et al., 2008). In *C. elegans*, *rps-24.2* has been shown to genetically interact with *let-7* (Ding et al., 2008) and RPS-0 has been found in association with AIN-2 (ALG- 1 interacting protein 2) (Zhang et al., 2007). RPS-14 was not detected in the AIN-2 associating complex probably because of a different proximity of RPS-14 to AIN-2. While the mechanism is still unclear, our findings point to a new direction in the study of miRNA function with the possibility that certain ribosomal proteins, like RPS-14, may interact with miRISC and play roles in miRNA-mediated translational repression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank C. Mello for providing the *gfp::alg-1* construct. We thank R. Ketting for the anti-DCR-1 antibody and M. Han for the anti-AIN-1 antibody. We thank R. Niwa and S. Roush for critical reading of this manuscript and all the Slack lab members for their support during the investigation. This work was supported by an NIH grants to F.S. (GM62594, GM64701), The Anna Fuller Fund Fellowship and The Anna D. Barker Fellowship in Basic Cancer Research (AACR) to S.-P.C.

References

Ambros V. The functions of animal microRNAs. Nature 2004;431:350–5. [PubMed: 15372042]

Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97. [PubMed: 14744438]

Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol 2007;23:175–205. [PubMed: 17506695]

Ceci M, Gaviraghi C, Gorrini C, Sala LA, Offenhäuser N, Marchisio PC, Biffo S. Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. Nature 2003;426:579–84. [PubMed: 14654845]

Chalfie M, Horvitz HR, Sulston JE. Mutations that lead to reiterations in the cell lineages of C. elegans. Cell 1981;24:59–69. [PubMed: 7237544]

Chan SP, Ramaswamy G, Choi EY, Slack FJ. Identification of specific *let-7* microRNA binding complexes in *Caenorhabditis elegans*. RNA 2008;14:2104–14. [PubMed: 18719242]

Chendrimada TP, Finn KJ, Ji X, Baillat D, Gregory RI, Liebhaber SA, Pasquinelli AE, Shiekhattar R. MicroRNA silencing through RISC recruitment of eIF6. Nature 2007;447:823–8. [PubMed: 17507929]

Ding L, Spencer A, Morita K, Han M. The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Mol Cell 2005;19:437–47. [PubMed: 16109369]

Ding XC, Slack FJ, Grosshans H. The *let-7* microRNA interfaces extensively with the translation machinery to regulate cell differentiation. Cell Cycle 2008;7:3083–90. [PubMed: 18818519]

Esquela-Kerscher A, Johnson SM, Bai L, Saito K, Partridge J, Reinert KL, Slack FJ. Post-embryonic expression of C. elegans microRNAs belonging to the lin-4 and let-7 families in the hypodermis and the reproductive system. Dev Dyn 2005;234:868–77. [PubMed: 16217741]

Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259–69. [PubMed: 16557279]

Eulalio A, Huntzinger E, Izaurralde E. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat Struct Mol Biol 2008;15:346–53. [PubMed: 18345015]

Fewell SW, Woolford. Ribosomal protein S14 of *Saccharomyces cerevisiae* regulates its expression by binding to *RPS14B* pre-mRNA and to 18S rRNA. Mol Cell Biol 1999;19:826–34. [PubMed: 9858605]

Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008;9:102–14. [PubMed: 18197166]

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell 2001;106:23–34. [PubMed: 11461699]

Hutvágner G, Simard MJ, Mello CC, Zamore PD. Sequence-specific inhibition of small RNA function. PLoS Biol 2004;2:E98. [PubMed: 15024405]

Ishizuka A, Siomi MC, Siomi H. A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev 2002;16:2497–2508. [PubMed: 12368261]

Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack F. RAS is regulated by the let-7 microRNA family. Cell 2005;120:635–47. [PubMed: 15766527]

Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J. Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature 2003;421:231–7. [PubMed: 12529635]

Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. Dev Cell 2006;11:441–50. [PubMed: 17011485]

Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell 1993;75:843–54. [PubMed: 8252621]

Maroney PA, Yu Y, Fisher J, Nilsen TW. Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat Struct Mol Biol 2006;13:1102–7. [PubMed: 17128271]

Mathonnet G, Fabian MR, Svitkin YV, Parsyan A, Huck L, Murata T, Biffo S, Merrick WC, Darzynkiewicz E, Pillai RS, Filipowicz W, Duchaine TF, Sonenberg N. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science 2007;317:1764–7. [PubMed: 17656684]

Mello CC, Kramer JM, Stinchcomb D, Ambros V. Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J 1991;10:3959–70. [PubMed: 1935914]

Miluzio A, Beugnet A, Volta V, Biffo S. Eukaryotic initiation factor 6 mediates a continuum between 60S ribosome biogenesis and translation. EMBO Rep 2009;10:459–65. [PubMed: 19373251]

Nilsen TW. Mechanisms of microRNA-mediated gene regulation in animal cells. Trends Genet 2007;23:243–9. [PubMed: 17368621]

Nilsson J, Sengupta J, Frank J, Nissen P. Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. EMBO Rep 2004;5:1137–41. [PubMed: 15577927]

Nottrott S, Simard MJ, Richter JD. Human *let-7a* miRNA blocks protein production on actively translating polyribosomes. Nat Struct Mol Biol 2006;13:1108–14. [PubMed: 17128272]

Olsen PH, Ambros V. The lin-4 Regulatory RNA Controls Developmental Timing in Caenorhabditis elegans by Blocking LIN-14 Protein Synthesis after the Initiation of Translation. Developmental Biology 1999;216:671–680. [PubMed: 10642801]

Petersen CP, Bordeleau ME, Pelletier J, Sharp PA. Short RNAs repress translation after initiation in mammalian cells. Mol Cell 2006;21:533–42. [PubMed: 16483934]

Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ. A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. Cell 2004;117:83–94. [PubMed: 15066284]

Pillai RS, Bhattacharyya SN, Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol 2007;17:118–26. [PubMed: 17197185]

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. Nature 2000;403:901–6. [PubMed: 10706289]

Robert F, Brakier-Gingras L. A functional interaction between ribosomal proteins S7 and S11 within the bacterial ribosome. J Biol Chem 2003;278:44913–20. [PubMed: 12937172]

Seggerson K, Tang L, Moss EG. Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene *lin-28* after translation initiation. Dev Biol 2002;243:215–25. [PubMed: 11884032]

Sengupta J, Nilsson J, Gursky R, Spahn CM, Nissen P, Frank J. Identification of the versatile scaffold protein RACK1 on the eukaryotic ribosome by cryo-EM. Nat Struct Mol Biol 2004;11:957–62. [PubMed: 15334071]

Standart N, Jackson RJ. MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. Genes Dev 2007;21:1975–82. [PubMed: 17699746]

Stefani G, Slack F. Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol. 2008

Sulston JE, Horvitz HR. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. Dev Biol 1977;56:110–56. [PubMed: 838129]

Tahbaz N, Kolb FA, Zhang H, Jaronczyk K, Filipowicz W, Hobman TC. Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. EMBO Rep 2004;5:189–94. [PubMed: 14749716]

Tasheva ES, Roufa DJ. Regulation of human *RPS14* transcription by intronic antisense RNAs and ribosomal protein S14. Genes Dev 1995;9:304–16. [PubMed: 7867928]

Thermann R, Hentze MW. Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature 2007;447:875–8. [PubMed: 17507927]

Timmons L, Fire A. Specific interference by ingested dsRNA. Nature 1998;395:854. [PubMed: 9804418]

Vella MC, Choi EY, Lin SY, Reinert K, Slack FJ. The *C. elegans* microRNA *let-7* binds to imperfect *let-7* complementary sites from the *lin-41* 3'UTR. Genes Dev 2004;18:132–7. [PubMed: 14729570]

Wakiyama M, Takimoto K, Ohara O, Yokoyama S. Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes Dev 2007;21:1857–62. [PubMed: 17671087]

Wang B, Yanez A, Novina CD. MicroRNA-repressed mRNAs contain 40S but not 60S components. Proc Natl Acad Sci USA 2008;105:5343–8. [PubMed: 18390669]

Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. Cell 1993;75:855–62. [PubMed: 8252622]

Yusupov MM, Yusupova GZ, Baucom A, Lieberman K, Earnest TN, Cate JH, Noller HF. Crystal structure of the ribosome at 5.5 A resolution. Science 2001;292:883–96. [PubMed: 11283358]

Zhang L, Ding L, Cheung TH, Dong MQ, Chen J, Sewell AK, Liu X, Yates JR, Han M. Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Mol Cell 2007;28:598–613. [PubMed: 18042455]

Zhou R, Hotta I, Denli AM, Hong P, Perrimon N, Hannon GJ. Comparative analysis of argonaute-dependent small RNA pathways in *Drosophila*. Mol Cell 2008;32:592–9. [PubMed: 19026789]

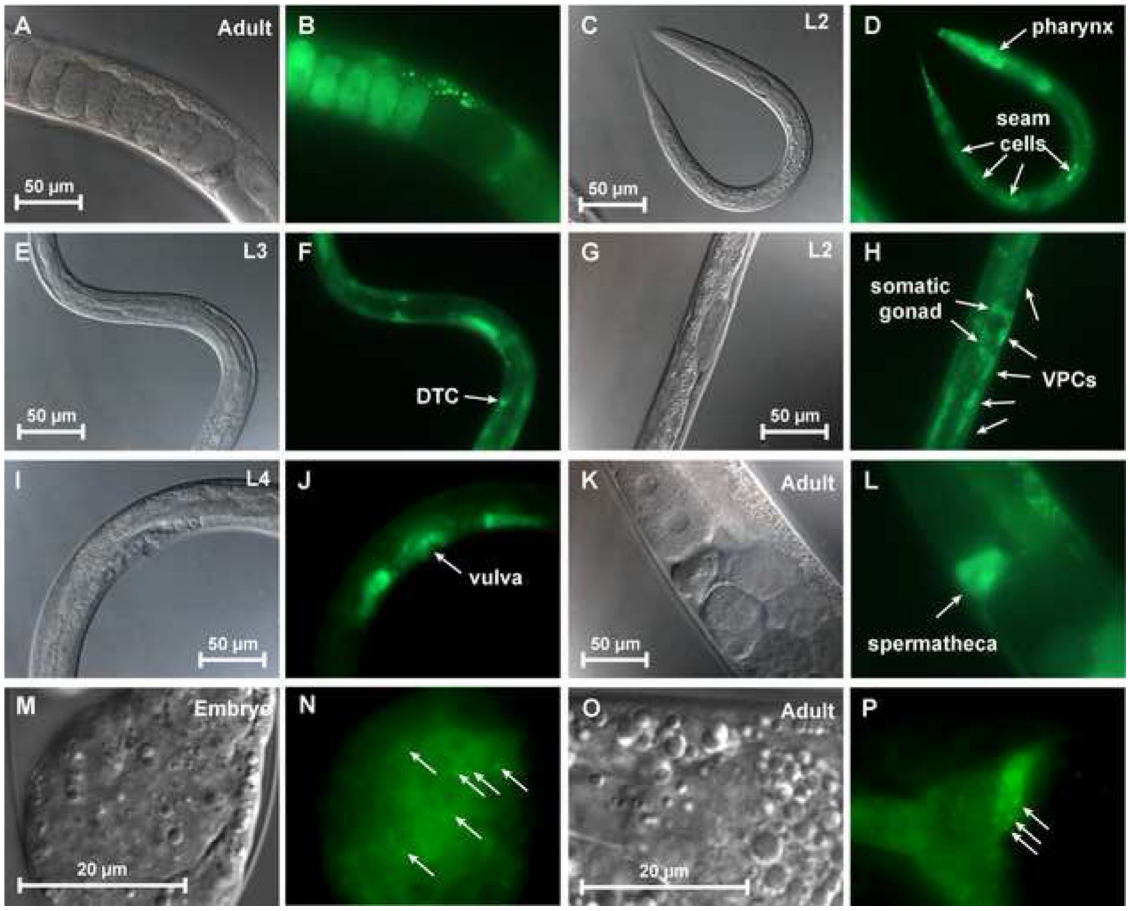

Figure 1.

gfp::alg-1 is expressed throughout *C. elegans*. *gfp::alg-1* is initially expressed in early embryos (A and B) and highly expressed in the pharynx and seam cells (C and D), distal tip cell (DTC) (E and F), somatic gonad and vulval precursor cells (VPCs) (G and H), vulva and spermatheca (I, J, K and L). The GFP::ALG-1 protein is localized in the cytoplasm and can be highly concentrated in sub-cellular granules, indicated by arrows in (N) an embryo at comma stage and (O) a cell in the tail of an adult animal.

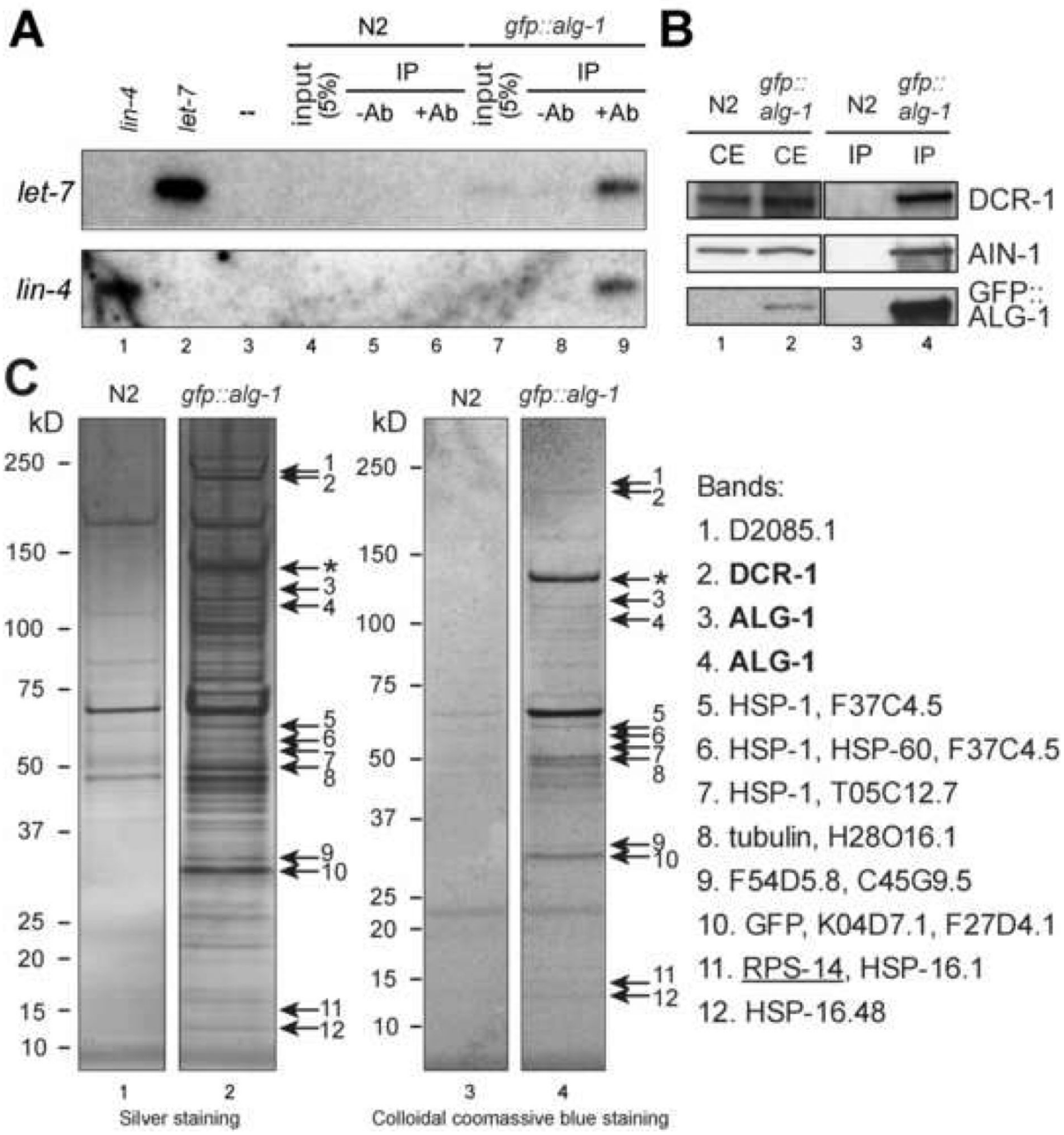

Figure 2.

Identification of ALG-1 interacting proteins. Total extracts prepared from animals carrying the integrated *gfp::alg-1* expression construct or from the N2 wild-type animals were immunoprecipitated with monoclonal anti-GFP antibody. (A) Immunoprecipitates were treated with proteinase K and total RNAs from the pellets were extracted and analyzed by northern blot. First two lanes are hybridizations to unlabeled *lin-4* and *let-7*, respectively. (B) Proteins eluted from the immunoprecipitates were separated on SDS-PAGE and analyzed by western blot. (C) Proteins eluted from the precipitates of a larger scale immunoprecipitation were divided to two equal aliquots and separated on two SDS-PAGE gels following by silver and colloidal coomassie staining, respectively. Twelve pieces containing interesting bands (indicated by numbers) were excised from the colloidal coomassie stained gel and sent for LC-

MS/MS sequencing. The names of proteins identified by the LC-MS/MS sequencing for each band are listed on the right. The names of RISC components are shown in bold. RPS-14 is highlighted with underline. The asterisk indicates GFP::ALG-1 band confirmed by western blot (data not shown).

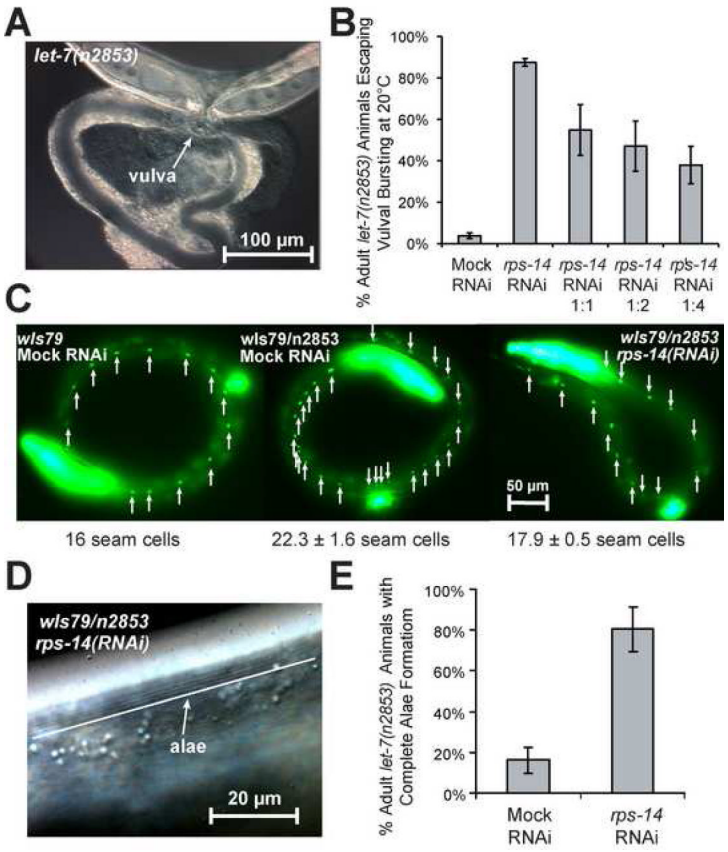

Figure 3.

rps-14(RNAi) suppresses *let-7(n2853)* mutant phenotypes. (A) The vulval bursting phenotype of a *let-7(n2853)* mutant animal at non-permissive temperature. The arrow indicates the burst vulva of the animal at the top of the image. (B) Vulva bursting was strongly suppressed by *rps-14(RNAi)* at ($n > 600$) and by RNAi with diluted bacteria food carrying *rps-14(RNAi)* plasmid (see Materials and Methods). (C) *wIs79 [ajm-1::gfp; scm-1::gfp]* animals showing seam cell nuclei at the L4-to-adult molt. Left, *wIs79* animals in a wild-type background under mock RNAi. Middle, *wIs79* animals in a *let-7(n2853)* background and under mock RNAi ($n = 110$). Right, *wIs79* in a *let-7(n2853)* background under *rps-14(RNAi)* ($n = 57$). Arrows indicate seam cell nuclei and the average numbers of nuclei are shown under the pictures. (D) Animals used in C were scored for complete alae formation. The picture shows complete alae in a *let-7(n2853)* animal under *rps-14(RNAi)*. (E) In *let-7(n2853)* animals, *rps-14(RNAi)* enhanced complete alae formation after the L4-to-adult to 80% of animals, compared to 16% under mock RNAi. Error bars show the standard error of the mean.

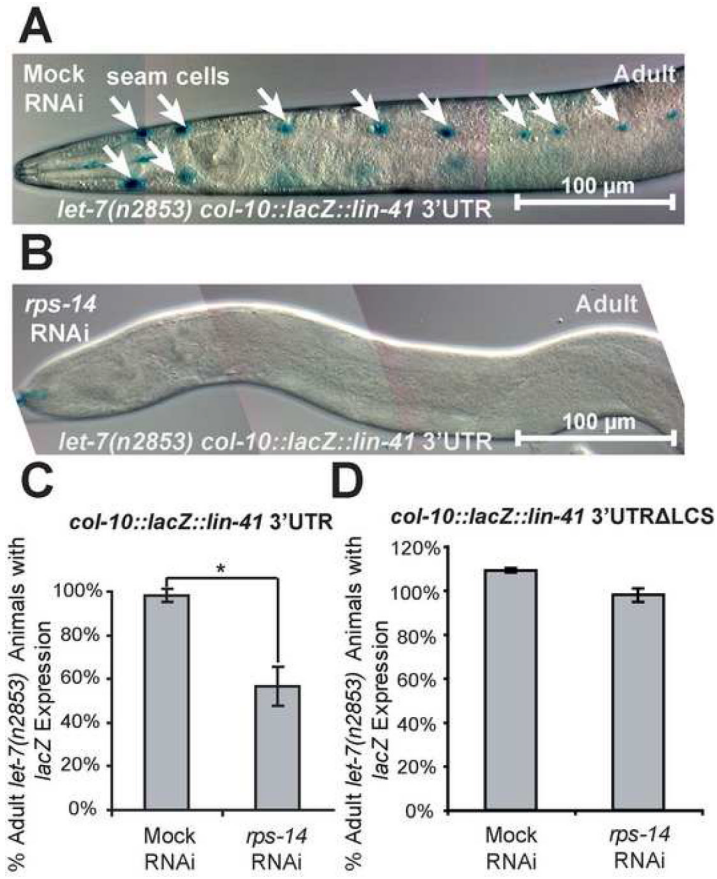

Figure 4.

RSP-14 modulates *let-7* function. (A) *let-7*-mediated repression of *col-10::lacZ::lin-41* 3′UTR reporter expression at the adult stage was defective in *let-7(n2853)* animals. Arrows indicated X-gal stained seam cell nuclei. (B) A representative of *let-7(n2853)* animals treated with *rps-14* RNAi. *let-7*-mediated repression of *col-10::lacZ::lin-41* 3′UTR reporter expression at the adult stage was restored and *lacZ* expression was reduced in seam cells. (C) 98% (n = 359) of *let-7(n2853)* animals maintained *lacZ* expression after the L4-to-adult molt. Under *rps-14 (RNAi)*, the relative percentage of adult animals (see Materials and Methods) with *lacZ* expression was reduced to 56% (n = 223). *, Student's t-test p = 0.011. (D) A *lacZ::lin-41* 3′ UTRΔLCS reporter was used for the control experiment. 109% (n = 321) and 98% (n = 225) of *let-7(n2853)* adult animals showed *lacZ* expression after the L4-to-adult molt under mock RNAi or *rps-14(RNAi)*, respectively.

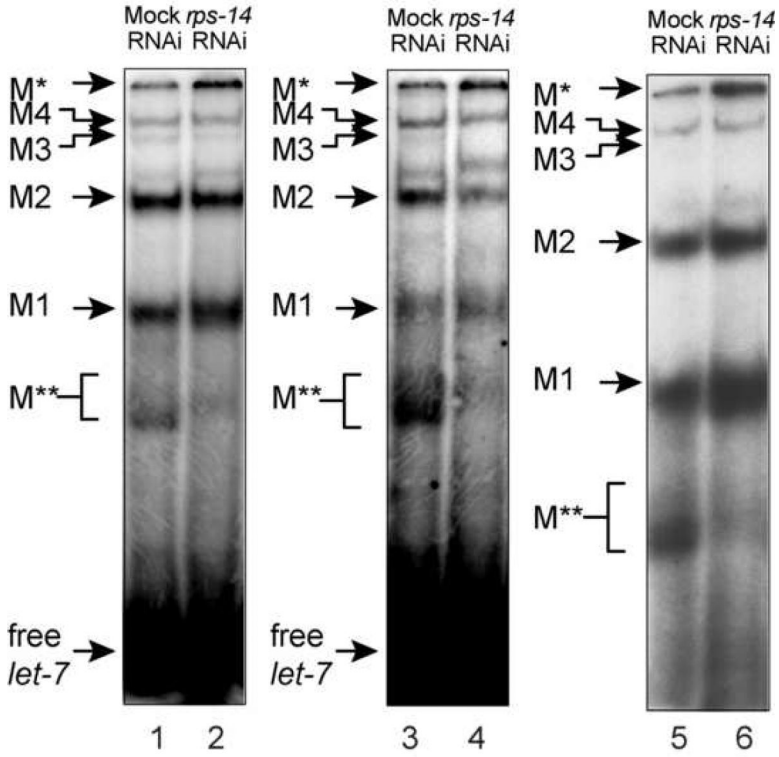

Figure 5.

rps-14(RNAi) altered *let-7* miRNP formation in cell extracts. Cytosolic cell extracts prepared from young adult wild-type animals treated with the control or *rps-14(RNAi)* were subjected to gel shift assay using radiolabeled *let-7* miRNA. Distinct *let-7* binding complexes are indicated (Chan et al., 2008). Three independent repeats are shown.