

# Regulation by *let-7* and *lin-4* miRNAs Results in Target mRNA Degradation

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## Summary

MicroRNAs (miRNAs) are ~22 nucleotide RNAs that negatively regulate the expression of protein-coding genes. In a present model of miRNA function in animals, miRNAs that form imperfect duplexes with their targets inhibit protein expression without affecting mRNA levels. Here, we report that in *C. elegans*, regulation by the *let-7* miRNA results in degradation of its *lin-41* target mRNA, despite the fact that its 3'UTR regulatory sequences can only partially base-pair with the miRNA. Furthermore, *lin-14* and *lin-28* are targets of the *lin-4* miRNA, and we show that the mRNA levels for these protein-coding genes significantly decrease in response to *lin-4* expression. This study reveals that mRNAs containing partial miRNA complementary sites can be targeted for degradation in vivo, raising the possibility that regulation at the level of mRNA stability may be more common than previously appreciated for the miRNA pathway.

## Introduction

MicroRNAs (miRNAs) comprise a new class of regulatory genes that function as 20–25 nucleotide (nt) single-stranded RNAs to control the expression of protein-coding genes (Bartel, 2004). Initially discovered as essential regulators of development in the nematode *Caenorhabditis elegans* (Pasquinelli and Ruvkun, 2002), miRNA genes are now believed to exist in all multicellular organisms. The RNA transcripts encoded by miRNA genes undergo multiple processing steps to produce the mature ~22 nt forms that downregulate protein expression of genes with antisense complementarity (Cullen, 2004). There are factors common to the RNA interference (RNAi) and miRNA pathways, yet the functional outcomes of these pathways seem to diverge in animals—RNAi results in mRNA degradation while miRNAs have been proposed to repress translation of their target mRNAs (Bartel, 2004).

Pioneering work in the Ambros and Ruvkun labs uncovered the first miRNA, *lin-4*, and its protein-coding target, *lin-14*, establishing a novel form of gene regulation (Lee et al., 1993; Wightman et al., 1993). The *lin-4* gene encodes a 21 nt RNA that can partially base-pair with multiple sites in the *lin-14* 3'UTR, causing inhibition of LIN-14 protein expression (Lee et al., 1993; Wightman et al., 1993). Early in the first larval (L1) stage, LIN-14 protein is present, but expression of *lin-4* miRNA

midway through L1 results in diminished protein levels (Olsen and Ambros, 1999; Wightman et al., 1991, 1993). The decrease in LIN-14 protein is partially dependent on *lin-4* activity and the *lin-4* complementary sites in the *lin-14* 3'UTR (Arasu et al., 1991; Wightman et al., 1991, 1993). Since the *lin-14* mRNA levels and polyribosome association appeared to be unaffected by *lin-4* regulation, the model emerged that miRNAs inhibit protein expression at the translational level (Olsen and Ambros, 1999; Wightman et al., 1993). Further genetic studies in *C. elegans* identified *lin-28* as another target of *lin-4* regulation mediated by a solitary complementary site in its 3'UTR (Moss et al., 1997). Subsequent analyses of *lin-28* mRNA levels and polyribosome association led to the conclusion that *lin-4* directed translational inhibition of this target mRNA as well (Moss et al., 1997; Seggerson et al., 2002).

The second miRNA gene to be discovered, *let-7*, regulates late larval development by inhibiting *lin-41* expression in at least some tissues (Reinhart et al., 2000; Slack et al., 2000). The *lin-41* mRNA contains two closely spaced *let-7* complementary sites (LCSs) that are predicted to form duplexes that include unpaired central regions. Fusion of the *lin-41* 3'UTR to reporter genes results in posttranscriptional regulation that is dependent on *let-7* miRNA and specific sequences and structures clustering at the LCS region (Reinhart et al., 2000; Slack et al., 2000; Vella et al., 2004a, 2004b). Based on the *lin-4/lin-14* paradigm, it was predicted that *let-7* recognition of LCS containing mRNAs would inhibit their translation (Slack et al., 2000).

Shortly after the discovery of *let-7* in worms, this gene was shown to be conserved broadly in many animal species (Pasquinelli et al., 2000). Homologs of *lin-41* have been predicted and, at least in some cases, they include potential LCSs (Pasquinelli et al., 2000; Slack et al., 2000). Hundreds of distinct miRNA genes are now known to exist in plants and animals, and intensive efforts have been made to match miRNAs with definitive targets. For animal miRNAs, these attempts are complicated by the fact that, except for a few rare exceptions, miRNA targets contain imperfect complementary sites. As such, hundreds of target mRNAs have been matched to a single given miRNA, and the vast majority of these predictions await experimental validation (Lai, 2004).

One model for miRNA function specifies that miRNAs negatively regulate the expression of mRNAs containing sites of complementarity; if the miRNA perfectly base-pairs with the target mRNA, then mRNA cleavage and degradation results, but if the miRNA:target duplex includes unpaired nucleotides, then translation of the mRNA is inhibited (Bartel, 2004). However, the mode of regulation can also be influenced by the type of protein complex recruited by a miRNA to its target site, as a recent report showed that specific miRNAs can facilitate AU-rich-mediated decay of unstable mRNAs (Jing et al., 2005). Additionally, transfection of exogenous miRNA duplexes into HeLa cells can cause moderate

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downregulation of multiple predicted target mRNAs (Lim et al., 2005).

Perfect base-pairing between a miRNA and its target site, which is very common in plants, results in cleavage of the mRNA between the tenth and eleventh positions of the duplex (Dugas and Bartel, 2004). Originally, this type of cleavage was shown to be directed by small interfering RNAs (siRNAs) during RNAi (Elbashir et al., 2001). Target RNAs designed to support perfect base-pairing with a miRNA can also be subjected to this type of cleavage, indicating that the duplex structure determines the mode of regulation and not the inherent nature of the guide RNA (Hutvagner and Zamore, 2002; Zeng et al., 2003). Recent studies in *Drosophila* and mammalian systems have identified Argonaute 2 (AGO2) as the enzyme responsible for the RNA-guided slicing activity (Liu et al., 2004; Meister et al., 2004). Much less is understood of how partially base-paired miRNA:target duplexes inhibit gene expression.

To explore the mechanism by which miRNAs regulate target mRNAs in vivo, we analyzed genetically defined endogenous targets of *let-7* and *lin-4* miRNAs in *C. elegans*. Unexpectedly, we found that *let-7* appears to promote the degradation of its *lin-41* target mRNA. This control is dependent on the LCS region and does not require perfect base-pairing between the miRNA and its target sites. Furthermore, we show that *lin-14* and *lin-28* mRNA levels decrease in response to *lin-4* miRNA. These results indicate that regulation of mRNA levels may contribute to the inhibition of protein expression mediated by the *lin-4* and *let-7* miRNAs during larval development in *C. elegans*.

## Results

### Expression of *lin-41* mRNA Is Developmentally Regulated

Genetic analyses in *C. elegans* indicate that *let-7* miRNA negatively regulates *lin-41* expression (Reinhart et al., 2000; Slack et al., 2000). A rescuing *lin-41*/GFP fusion protein is downregulated upon the expression of *let-7* miRNA in the later larval stages of development (Slack et al., 2000). To test if endogenous *lin-41* mRNA levels are subject to regulation by *let-7*, developmentally staged preparations of RNA from wild-type worms were analyzed by Northern blot experiments. We observed a significant decline in *lin-41* mRNA levels during the later larval stages of development (Figure 1A). The decrease in *lin-41* mRNA correlates with the appearance of mature *let-7* miRNA, which begins in the third larval stage (L3; Figure 1A; Bracht et al., 2004; Johnson et al., 2003; Reinhart et al., 2000), opening the possibility that *let-7* regulates this target by promoting its destabilization. This observation was surprising given the current model that miRNA target transcripts are usually not subject to degradation in animals (Bar- tel, 2004).

### Reduction of *lin-41* mRNA Levels Is Dependent on *let-7* miRNA

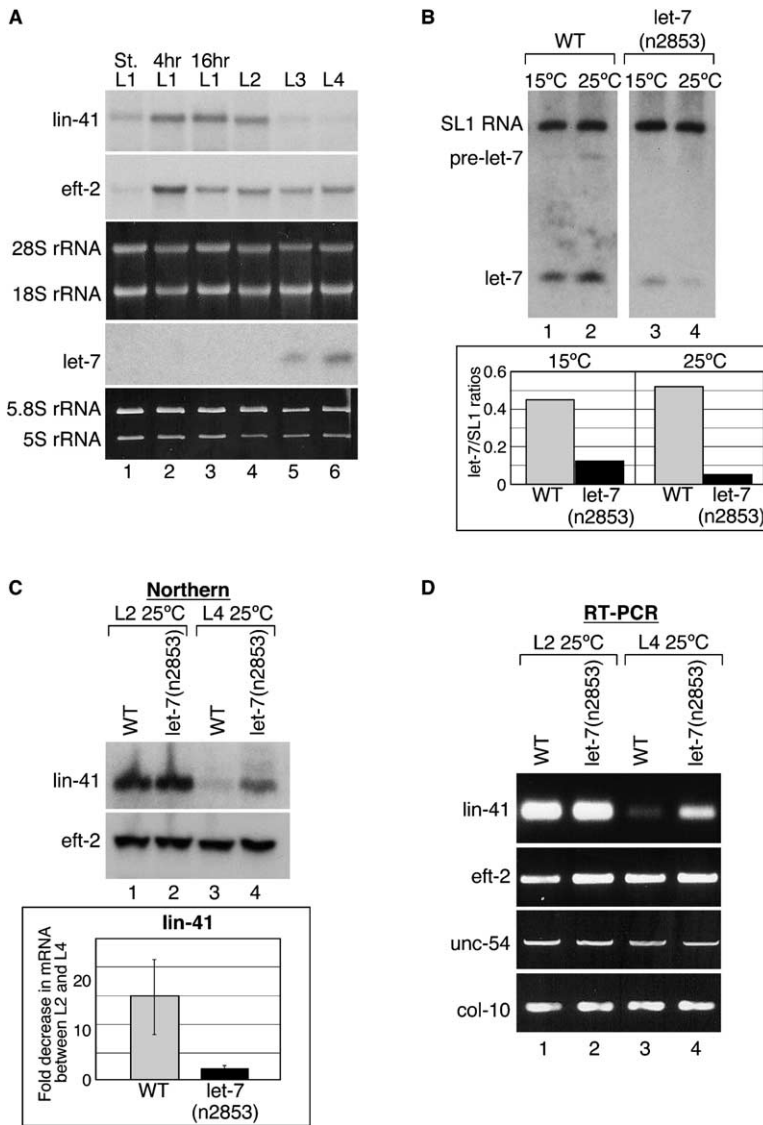
To test if the decrease in *lin-41* mRNA is dependent on *let-7*, we analyzed *lin-41* mRNA levels in worms deficient in *let-7* gene activity. The *let-7(n2853)* mutation

causes heterochronic defects in worm development, the severity of which is temperature sensitive (Reinhart et al., 2000). This *let-7* allele contains a point mutation that changes guanine to adenine at the fifth base position of the mature sequence of the miRNA, which is predicted to disrupt duplex formation with the target sites in the *lin-41* 3' UTR (Reinhart et al., 2000; Slack et al., 2000). Additionally, this base change results in significantly lower levels of the mature miRNA; Northern analyses showed that *let-7* levels at the L4 stage in the mutant were lower by over 3-fold at 15°C and over 10-fold at 25°C compared to wild-type (Figure 1B). The cause for this difference in relative mature *let-7* RNA levels is presently unknown.

Northern and RT-PCR analyses using these same RNA samples revealed that there is an inverse correlation between *lin-41* mRNA and *let-7* miRNA expression (Figures 1C and 1D; see Table S1 in the Supplemental Data available with this article online). We detect a greater than 3-fold higher level of *lin-41* in RNA samples collected from *let-7(n2853)* compared to wild-type worms at the L4 stage (Figure 1C, lanes 3 and 4). Relative *lin-41* mRNA levels were indistinguishable between wild-type and *let-7(n2853)* worms at the L2 stage (Figures 1C and 1D), which is prior to the onset of *let-7* expression (Figure 1A). Comparable results were obtained using primers covering open reading frame (ORF) or 3' UTR sequences for RT-PCR analyses (Figures 1D and S1A and data not shown). The expression levels of control mRNAs not predicted to be regulated by *let-7*, including *eft-2*, *unc-54* and *col-10*, were indistinguishable in wild-type versus *let-7(n2853)* worms during larval development (Figure 1D). The results of quantitative real time PCR (qRT-PCR) of RNA samples collected from wild-type and *let-7(n2853)* worms further support our conclusion that *let-7* activity is important for a significant decrease in *lin-41* mRNA levels in the late larval stages of worm development (Table S1).

### The *lin-41* 3' UTR Directs Downregulation of mRNA Expression

The LCS region of the *lin-41* 3' UTR mediates *let-7*-dependent regulation of reporter genes (Reinhart et al., 2000; Slack et al., 2000; Vella et al., 2004a, 2004b). To test if such transgenes are subject to control at the level of mRNA stability, we analyzed mRNA levels of the transgene-encoding LacZ fused to the *lin-41* 3' UTR driven by the *col-10* promoter (Figure 2A). We observed that the levels of the LacZ mRNA containing the *lin-41* 3' UTR (+LCS) dramatically decreased between the L2 and L3 stages (Figure 2B, lanes 1 and 2). An approximately 3-fold decrease in LacZ fusion mRNA levels between the L2 and L3 stages was determined by qRT-PCR (Figure 2C). Regulation of this reporter is lost by deletion of the LCS ( $\Delta$ LCS) region of the *lin-41* 3' UTR (Reinhart et al., 2000; Slack et al., 2000; Vella et al., 2004a, 2004b). We find that mRNA levels of this  $\Delta$ LCS construct appear unchanged from the L2 to L3 stages as determined by standard or qRT-PCR analyses (Figures 2B and 2C), indicating that the LCS region is important for developmentally regulated reduction of the mRNA.



**Figure 1. *let-7* miRNA Developmentally Regulates *lin-41* mRNA Expression**

(A) Total RNA from developmentally staged wild-type worms was isolated from starved L1 (St. L1)-, 4 hr and 16 hr on food L1-, and L2-, L3-, and L4-stage worms. RNA samples were separated under denaturing conditions by 11% PAGE or 1% agarose gel electrophoresis and probed by Northern analyses for mature *let-7* or *lin-41* (3' UTR probe, see Figure S1A). Reprobing of the blot for *eft-2* mRNA and ribosomal RNAs (rRNAs) detected by ethidium bromide staining of the gels serve as controls.

(B) RNA from L4-stage wild-type (wt) and *let-7(n2853)* worms cultured at 15°C and 25°C was analyzed for *let-7* RNA expression by PAGE Northern analyses. A probe complementary to wt *let-7* sequence was used for wt samples (lanes 1 and 2) and a probe complementary to the *let-7(n2853)* point mutation was used for the mutant samples (lanes 3 and 4). A probe for SL1 RNA was included as a control for comparison of relative *let-7* RNA levels in wt versus mutant worms. Phosphorimager analysis was used to quantify the relative levels of RNA, and the ratios of *let-7* to SL1 RNA in each sample are shown in the graphs.

(C) RNA from L2- and L4-stage wt and *let-7(n2853)* worms cultured at 25°C was analyzed for *lin-41* mRNA levels as described in (A). The blot was reprobed for *eft-2* mRNA, and the average fold decrease and standard deviations in *lin-41* mRNA levels from L2 to L4 stage after normalization to *eft-2* levels from three independent experiments is shown.

(D) RNA samples from L2- and L4-stage wt and *let-7(n2853)* worms cultured at 25°C were subjected to reverse transcription followed by PCR with primers that span the LCS region (A465 + A410, see Figure S1A) of *lin-41* mRNA or that detect the control mRNAs *eft-2*, *unc-54*, and *col-10*. The products were separated by electrophoresis in 1% agarose gels and visualized by ethidium bromide staining.

### Downregulation of *lin-41* Is Posttranscriptional

The dramatic decrease in *lin-41* mRNA levels during development does not appear to be associated with transcriptional repression of this gene. To analyze the transcriptional state of the *lin-41* gene, we performed RNA polymerase II chromatin immunoprecipitation (ChIP) experiments. The human  $\alpha$ -RNA Pol II antibodies reacted with the *C. elegans* protein (Figure 3A) and were used for ChIP experiments to compare Pol II association with the *lin-41* gene at the L2 and L4 stages of development. To rule out the possibility of the polymerase stalled at promoter sequences, we used PCR primers within the coding region of the analyzed genes (Sandoval et al., 2004). These experiments showed similar levels of *lin-41* sequence bound to RNA Pol II at the L2 and L4 stages (Figure 3B). The *lin-41* signal detected from  $\alpha$ -Pol II- (+) versus the mouse preimmune serum (-)-precipitated DNA was comparable to that of the control mRNA gene, *col-10*, but the Pol I transcribed rRNA gene was not enriched in the  $\alpha$ -Pol II precipitate

(Figure 3B). These results indicate that *lin-41* is being transcribed in vivo at the L4 stage. Taken together with the finding that *let-7* activity regulates the level of *lin-41* or LCS-containing reporter mRNAs, we conclude that a miRNA-directed posttranscriptional mechanism is responsible for the dramatic decrease in *lin-41* mRNA during larval development.

### Accumulation of *lin-41* mRNA Degradation Products

To explore the mechanism by which *let-7* directs downregulation of its target mRNA, we attempted to identify specific degradation products for *lin-41*. In longer exposures of our Northern analyses of *lin-41* mRNA, we detected a faint, but reproducible, ~500 nt band specifically in RNA samples from L4-stage wild-type worms. Probes specific for sequences downstream, but not upstream, of the LCS region (see Figure S1A) hybridize to this band (Figure 4A). Appearance of the band was dependent on *let-7* activity and correlated with the

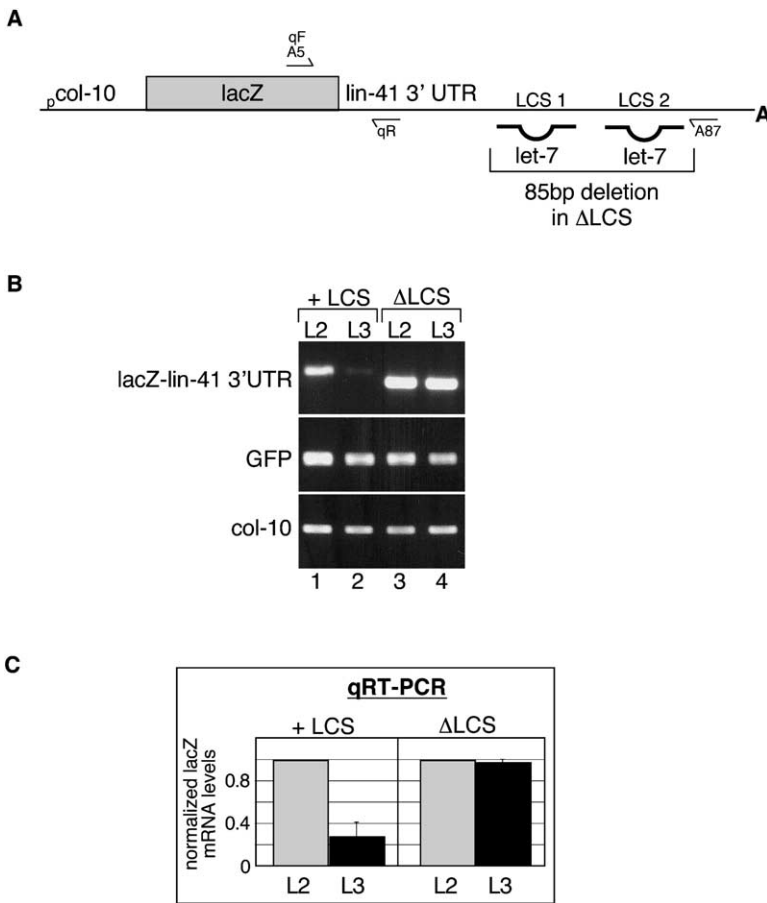


Figure 2. The *lin-41* 3' UTR Regulates Reporter-Gene Expression

(A) Schematic of LacZ/*lin-41* 3' UTR reporter constructs with (+LCS) and without the 85 base pairs of LCS region ( $\Delta$ LCS; Reinhart et al., 2000; Slack et al., 2000). The relative positions of primers used in qRT-PCR (qF and qR) and in standard RT-PCR are indicated by horizontal arrows.

(B) RNA was isolated from strains containing the lacZ/*lin-41* 3' UTR constructs at the L2 and L3 stages of development. RT-PCR analyses were used to detect transgene-encoded RNA containing the lacZ-*lin-41* 3' UTR sequence. Detection of GFP mRNA, expressed from the *goa-1::GFP* marker coinjected with the +LCS and  $\Delta$ LCS constructs (Reinhart et al., 2000; Slack et al., 2000), and endogenous *col-10* mRNA are shown as controls.

(C) Quantification of the relative levels of lacZ-*lin-41* 3' UTR mRNA levels by qRT-PCR. GFP mRNA levels from the coinjected marker gene were used for normalization, and L2 stage levels were set to 1.0 for calculation of fold modulation. The error bars indicate the standard deviation from two replicates of qRT-PCR experiments.

stage-specific decrease in full-length *lin-41* mRNA levels (Figure 4A), suggesting that it was a 3' mRNA degradation product resulting from *let-7*-mediated regulation. Prolonged exposures of the same blots re-probed for control mRNAs *eft-2* or *unc-54* revealed no stage or *let-7*-dependent bands (data not shown).

To test if the ~500 nt *lin-41* RNA species was a substrate for 5'-3' exonucleases, which recognize exposed 5'-monophosphates on decapped mRNAs or decay intermediates, we performed RNAi targeting the

two predicted 5'-3' exonucleases in *C. elegans* (Newbury and Woollard, 2004). The RNAi hypersensitive strain *rrf-3* (Simmer et al., 2002) was used for feeding RNAi experiments targeting *xrn-1* or Y48B6A.3. RNA isolated from worms grown on bacteria expressing dsRNA against Y48B6A.3 showed a dramatic reduction in mRNA levels of this targeted gene as well as a cross effect on the homologous *xrn-1* gene, but not on other control mRNAs (Figure S2), and thus results in a general reduction in 5'-3' exonuclease gene expression. RNAi

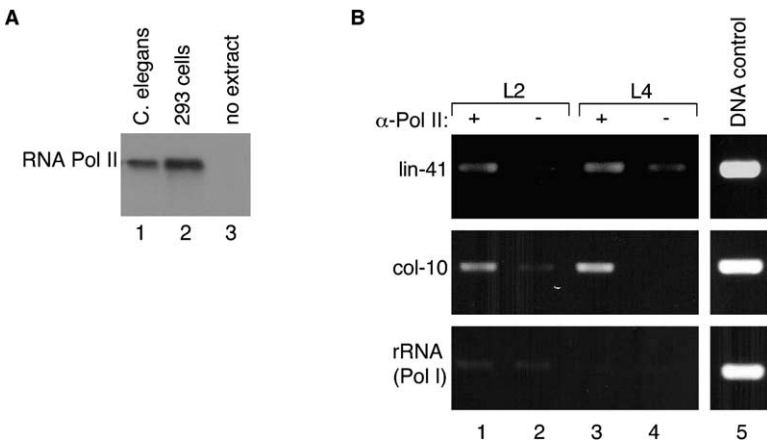


Figure 3. ChIP Analysis of *lin-41* Expression

(A) Protein extracts from *C. elegans* and 293 cells and buffer control were separated by SDS-PAGE and probed for RNA Polymerase II by Western blot analyses.

(B) ChIP experiments were performed to analyze the association of RNA polymerase II with transcribed regions of *lin-41* and the positive control *col-10* mRNA genes and the negative control 5.8S RNA polymerase I gene at the L2 and L4 stages of development. The PCR products resulting from the  $\alpha$ -RNA Pol II (+) and control mouse preimmune serum (-) immunoprecipitates were separated in 1% agarose gels and visualized by ethidium bromide staining. The DNA control (lane 5) shows PCR products generated using the same primers with purified genomic *C. elegans* DNA.

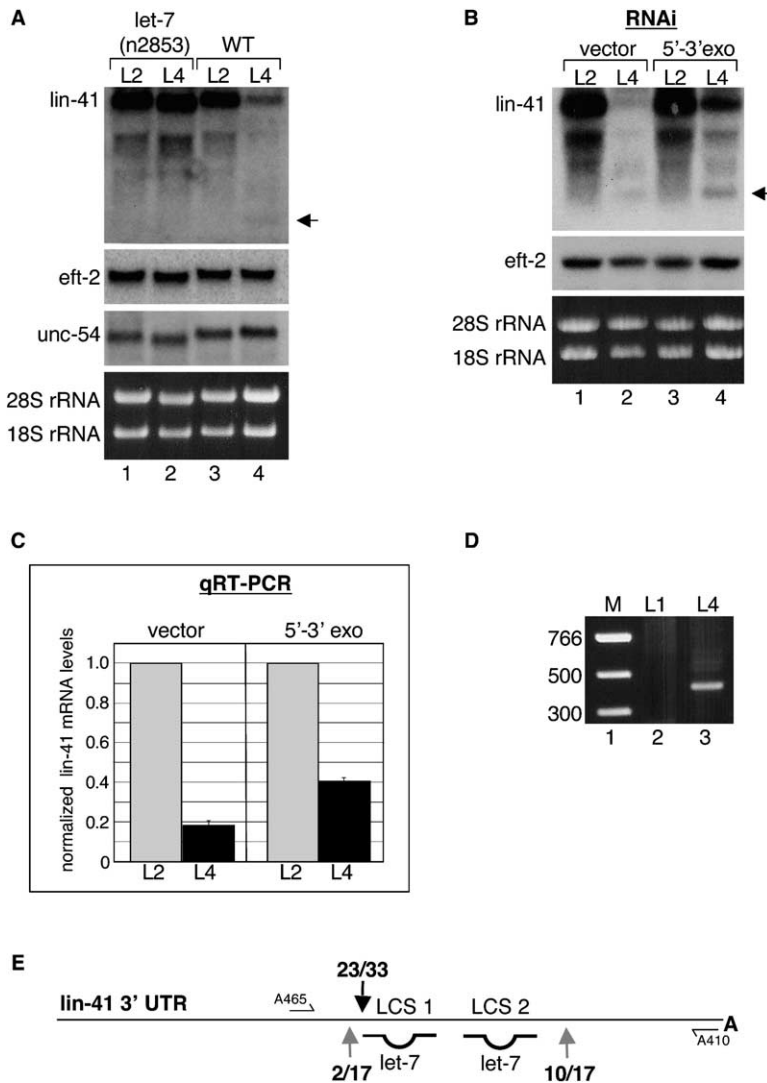


Figure 4. Identification of *lin-41* Degradation Products

(A) Northern analyses of total RNA from *let-7(n2853)* and wt worms cultured at 25°C were performed as described in Figure 1 except that the probe only included sequences downstream of the LCS region of the *lin-41* 3' UTR (see Figure S1A). The blot was exposed to film three times longer than usual to detect the specific ~500 nt degradation product indicated by the arrow. Panels of the blot reprob for *eft-2* and *unc-54* and ethidium bromide staining of rRNAs are shown as controls.

(B) RNA was isolated from worms fed vector control or Y48B6A.3 dsRNA expressing bacteria at the L2 and L4 stages and analyzed as described in (A). An arrow indicates the degradation product that accumulates upon depletion of the 5'-3' exonucleases.

(C) Quantification of the relative levels *lin-41* mRNA levels by qRT-PCR. The *eft-2* mRNA levels were used for normalization and L2 stage levels were set to 1.0 for calculation of fold modulation (see Experimental Procedures). The error bars indicate the standard deviation from two replicates of qRT-PCR experiments.

(D) RNA from wt L1- and L4-stage worms was subjected to RNA oligonucleotide-mediated 5' RACE. A major *lin-41* RACE product was amplified from L4- but not L1-stage samples. Lane 1 is the New England Biolabs PCR DNA size marker.

(E) Cloning of PCR-amplified *lin-41* 5' and 3' RACE products revealed 3' degradation products that primarily mapped to the beginning of LCS1 (black, downward arrow) and 5' products that primarily mapped ~40 nt downstream of LCS2 and 2/17 that were located 8 nucleotides upstream of LCS1 (gray, upward arrows). The relative positions of the *lin-41* forward primer (A465) used to clone the 3' RACE products and the reverse primer (A410) used to clone the 5' RACE products are indicated by horizontal arrows.

against these genes caused accumulation of the ~500 nt *lin-41* degradation product (Figure 4B). After normalization to the *eft-2* control mRNA, the band was estimated to be 2-fold enriched in worms depleted of 5'-3' exonuclease activity. The ~500 nt degradation product also accumulated when RNAi against the 5'-3' exonucleases was carried out in wild-type but not in *let-7(n2853)* worms (data not shown), indicating that its production is *let-7* dependent.

We also noted a marked increase in the level of apparently full-length *lin-41* mRNA in worms undergoing RNAi against the 5'-3' exonucleases compared to the vector control (Figures 4B and 4C), RNAi targeting *xrn-1* alone, or RNAi against the nonsense-mediated decay (NMD) gene *smg-2* (data not shown). Additionally, no apparent differences from wild-type in *lin-41* mRNA regulation were detected in RNA collected from *smg-2(r908)* genetic mutant worms, which are deficient for the NMD pathway (Page et al., 1999), or from *rde-1(ne300)* or *rde-4(ne299)* mutant worms, which are fully RNAi defective (Tabara et al., 1999; Figure S3). Normal regulation of *lin-41* mRNA, in the *smg-2*, *rde-1*, and *rde-4*

mutants was expected, as these mutants do not display heterochronic phenotypes that would be expected from compromised *let-7* function. Although *lin-41* mRNA is inefficiently downregulated in worms undergoing RNAi against the 5'-3' exonucleases (Figures 4B and 4C), these worms display no obvious *let-7*-like heterochronic phenotypes in our assays. These results are consistent with a role for 5'-3' exonuclease activity downstream of miRNA action to complete the degradation of decapped or endonucleolytic cleaved mRNAs that are no longer substrates for translation, comparable to the role of this activity in the RNAi pathway (Orban and Izaurralde, 2005; Souret et al., 2004).

The effect of RNAi against the 5'-3' exonuclease genes was specific for *lin-41* RNA at the L4 stage. Quantification of full-length *lin-41* mRNA levels detected by Northern blotting (Figure 4B) indicates a 4-fold reduction in mRNA upon depletion of 5'-3' exonuclease activity compared to the 12-fold decrease from L2 to L4 in vector control samples. Importantly, no difference was detected in *lin-41* mRNA levels for vector versus RNAi against the 5'-3' exonuclease genes

at the L2 stage nor for the *eft-2* control mRNA at either of the stages tested. The difference in *lin-41* mRNA levels specifically at the L4 stage in the RNAi experiment was also supported by qRT-PCR analyses, which indicated a 2-fold increase in full-length *lin-41* mRNA levels in worms subjected to RNAi against the 5'-3' exonucleases compared to the control (Figure 4C). Taken together, these data indicate that efficient degradation of *lin-41* mRNA after targeting by *let-7* miRNA requires 5'-3' exonuclease activity.

The *let-7* dependent ~500 nt *lin-41* band that accumulated upon depletion of 5'-3' exonuclease activity (Figures 4A and 4B) was predicted to contain a 5' monophosphate, which could result from endonucleolytic cleavage or stalled 5'-3' decay of the mRNA. To identify the degradation product, we subjected L4 stage RNA from wild-type worms directly to RNA oligonucleotide ligation, followed by oligonucleotide (dT)-primed reverse transcription and PCR using a reverse primer complementary to sequences upstream of the *lin-41* polyadenylation signal (Figure S1A). This reaction primarily yielded an ~400 bp product, which was undetectable when the same experiment was performed with L1-stage RNA (Figure 4D). The PCR products resulting from reverse transcription of L4-stage RNA was used for cloning and subsequent sequencing, which showed that 23 of 33 clones terminated at the 5' end of the first LCS in the *lin-41* 3' UTR (Figure 4E). The other ten sequences mapped to scattered positions throughout the 3' UTR. Comparable results (8/12 clones) were obtained when the cloning experiment was repeated with L4-stage RNA from *rrf-3* worms subjected to RNAi against the 5'-3' exonucleases.

The *lin-41* mRNA fragments that map to the beginning of LCS1 could result from specific cleavage at this position or from upstream cleavage events followed by mRNA decay to the predicted LCS1-*let-7* duplex. If initial cleavage is directed to this site, then 5' fragments should also map to this position. We ligated the 5'-phosphorylated RNA oligonucleotide to L4-stage RNA and performed reverse transcription by priming the RNA oligonucleotide sequence. Subsequent PCR amplification and cloning revealed 5' fragments that mapped primarily (10/17) to a position ~40 nucleotides downstream from LCS2; two other clones mapped a few nucleotides upstream of the major 3' site at LCS1 (Figure 4E). No fragments were identified that mapped within the LCS region, indicating that it may be protected in vivo. Since the 3' and 5' degradation products do not point to a common cleavage site, it is likely that they are decay intermediates that could result when the degradation machinery encounters the LCS region. Furthermore, accumulation of apparently full-length *lin-41* mRNA upon depletion of 5'-3' exonuclease activity (Figure 4B) is consistent with 5'-3'-mediated decay initiating at the beginning of decapped mRNA. We conclude that regulation by *let-7* miRNA results in degradation of its target mRNA and the mechanism involves the generation of substrates for 5'-3' exonuclease activity.

#### The *lin-4* miRNA Causes Decreased Levels of Its mRNA Targets

The unexpected finding that *let-7* miRNA appears to promote destabilization of its target mRNA prompted

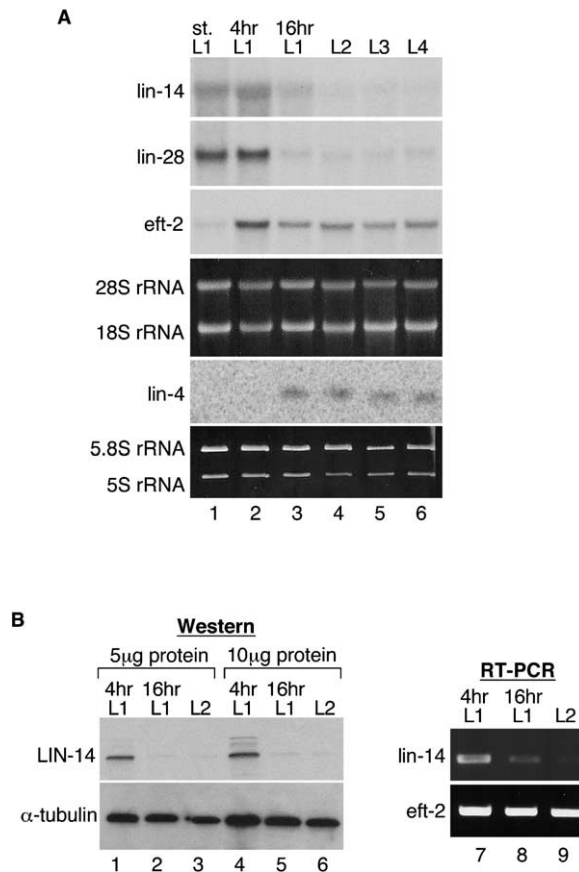


Figure 5. Temporally Regulated *lin-14* and *lin-28* Gene Expression (A) Developmentally staged RNA samples were analyzed for *lin-4*, *lin-14*, and *lin-28* RNA expression by reprobing blots shown in Figure 1A. Identical results were obtained with probes for *lin-28* that covered the 3' UTR only or the entire gene and with probes for *lin-14* that covered the 3' UTR or exons 7–13 (see Figures S1B and S1C). The wild-type *lin-14* mRNA typically does not appear as a discrete band, probably because of the three mRNA isoforms for this gene (Wightman et al., 1991). (B) Worms were staged as indicated and collected for analyses of *lin-14* protein (lanes 1–6) and RNA (lanes 7–9) expression. Samples of 5 µg and 10 µg total protein were subjected to Western blot analysis of LIN-14 and the control α-tubulin proteins. RNA samples from the same worm populations were used for RT-PCR experiments to compare relative expression levels of *lin-14* mRNA and the *eft-2* mRNA control.

us to test if other established miRNA targets are subject to this type of regulation. The *lin-14* and *lin-28* genes contain sites in their 3' UTRs that are complementary to *lin-4*, and these sites mediate negative regulation by this miRNA (Ha et al., 1996; Moss et al., 1997; Wightman et al., 1993). In contrast to previous reports (Moss et al., 1997; Olsen and Ambros, 1999; Seggerson et al., 2002; Wightman et al., 1993), we observed a significant decrease in *lin-14* and *lin-28* mRNA levels at the point in development when *lin-4* miRNA first appears (Figure 5A). These mRNAs are readily detectable in early L1 and then diminish by late L1 and remain low throughout the rest of larval development.

The level of LIN-14 protein dramatically decreases from the L1 to the L2 stage (Olsen and Ambros, 1999;

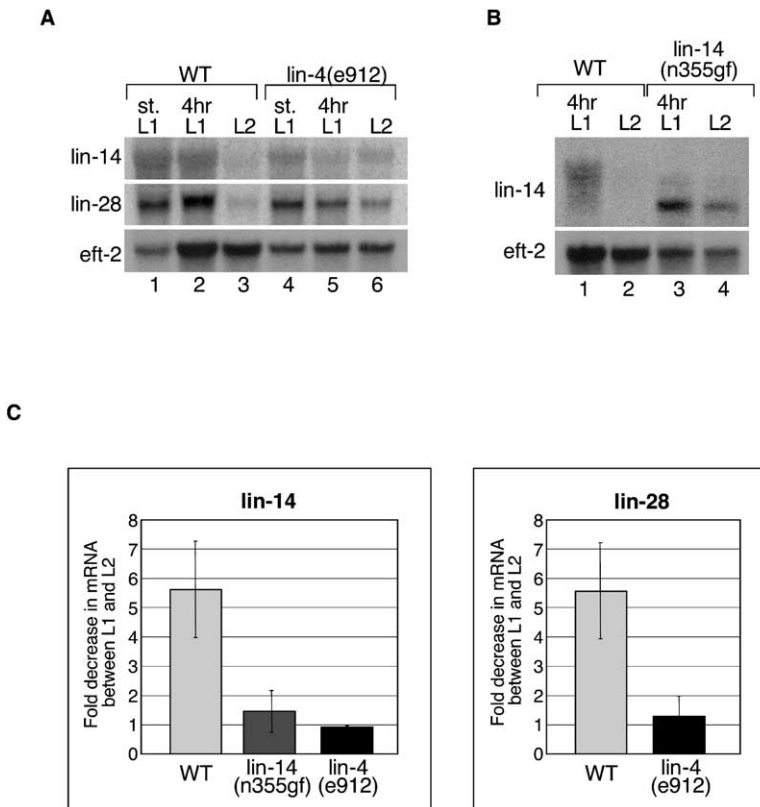


Figure 6. *lin-4* miRNA Developmentally Regulates Target mRNA Expression

(A) RNA from starved L1 (St. L1)- and fed 4hr L1- and L2-stage wt and *lin-4(e912)* worms was analyzed for *lin-14*, *lin-28*, and control *eft-2* mRNAs by Northern analyses.

(B) RNA from wt or *lin-14(n355gf)* mutant worms was probed for *lin-14* mRNA by Northern analyses, using the ORF probe which covers sequences present in wt and the *lin-14(n355gf)* deletion mutant (see Figure S1B). The lesion in the *lin-14(n355gf)* mutant results in ~2.2 kb bands instead of the ~3.5 kb bands produced by the wt allele (Wightman et al., 1991).

(C) Phosphorimager analyses of Northern blots were used to normalize *lin-14* or *lin-28* mRNA levels to the *eft-2* control and the relative change in target mRNA levels between the fed L1 to L2 stages are indicated in the histograms, which represent the averages and standard deviations from two or more independent RNA samples for each.

Wightman et al., 1993). To compare the timing of changes in *lin-14* protein and mRNA levels, we synchronized wild-type worm populations and split samples for RNA or protein analyses at 4 hr and 16 hr postfeeding-L1 stage and the mid-L2 stage. As previously observed, LIN-14 protein is present at L1 stage prior to *lin-4* expression but becomes virtually undetectable by the end of this stage (Figure 5B, lanes 1–6; Olsen and Ambros, 1999; Wightman et al., 1993). RT-PCR analyses of RNA from these same populations of worms showed that *lin-14* mRNA levels diminished in a pattern similar to that of LIN-14 protein expression (Figure 5B, lanes 7–9). Taken together, there does not appear to be a substantial delay between the downregulation of *lin-14* protein and mRNA levels.

The decrease in *lin-14* and *lin-28* mRNA levels at the L2 stage is dependent on *lin-4* miRNA. The *lin-4(e912)* lesion deletes the miRNA sequence, and several cell types in worms containing this mutation fail to develop beyond the first larval-stage fates (Lee et al., 1993). To test if *lin-14* or *lin-28* mRNA levels are affected by *lin-4* activity, we collected RNA from developmentally staged wild-type and *lin-4(e912)* mutant worms and performed Northern analyses (Figure 6A). Compared to the greater than 5-fold decrease in *lin-14* and in *lin-28* mRNA levels in wild-type worms, the levels of these mRNAs remained nearly constant from the L1- to the L2-stage in *lin-4(e912)* worms (Figure 6C). Quantification of *lin-14* and *lin-28* mRNA levels by qRT-PCR yielded similar results (Table S1). We note that the fold decrease in *lin-14* mRNA levels is consistent with the 4-fold reduction in LIN-14 protein levels mediated by *lin-4* miRNA (Wightman et al., 1993).

The *lin-4* complementary sites in the *lin-14* 3' UTR are important for decreased mRNA expression. The *lin-14(n355gf)* allele contains a breakpoint mutation in the *lin-14* 3' UTR that removes all predicted *lin-4* complementary sites and is associated with failure to downregulate LIN-14 protein expression at the L2 stage (Wightman et al., 1991, 1993). Northern and qRT-PCR analyses of RNA collected from *lin-14(n355gf)* worms indicated that mRNA expression from this mutant allele was not subject to the same level of downregulation observed in wild-type worms from the L1 to L2 stages (Figures 6B and 6C; Table S1). These results show that developmentally regulated expression of *lin-14* mRNA is dependent on 3' UTR sequences. Taken together, a significant outcome of *lin-4* recognition of 3' UTR complementary sites appears to be downregulation of target mRNA levels.

## Discussion

A general model for gene regulation by miRNAs holds that partial base-pairing between a miRNA and its target mRNA results in translational repression without destabilization of the mRNA. However, there is some controversy over this proposal, and here we present evidence that mRNA degradation does occur in vivo in the context of the *C. elegans let-7* and *lin-4* miRNAs and their targets, which contain recognition sequences of imperfect complementarity. We show that *lin-41* or transgene reporter mRNAs containing the *lin-41* 3' UTR are downregulated, and the degradation is dependent on the *let-7* miRNA and the LCS region. Additionally, we present evidence that the *lin-14* and *lin-28* targets

of the founding miRNA gene, *lin-4*, are also subject to reduction at the mRNA level. Although it remains possible that translational regulation precedes degradation of these miRNA targets, our results argue against the simple model that mRNA fate is entirely dependent upon the degree of base-pairing with a miRNA.

The model for translational control by ~22 nt RNAs of genes containing imperfect complementary sites initiated from analyses of the genetically defined targets of *lin-4* regulation in *C. elegans*. Because the deletion of elements conserved between *C. elegans* and *C. briggsae* in the *lin-14* 3' UTR results in misregulation of LIN-14 protein expression, it was concluded that these UTR regions are important for posttranscriptional control of this gene (Wightman et al., 1991, 1993). Genetic experiments pointed to *lin-4* as a negative regulator of *lin-14* expression (Ambros, 1989; Arasu et al., 1991), and the discovery that *lin-4* encodes a 21 nt RNA that can base-pair with elements in conserved regions of the *lin-14* 3' UTR supported the model that *lin-4* directly interacted with the *lin-14* 3' UTR regulatory sequences to inhibit protein expression (Lee et al., 1993; Wightman et al., 1993).

Originally, it was concluded that *lin-4* inhibited translation of *lin-14* by an unknown mechanism. This model was based primarily on data indicating that the relative level of *lin-14* mRNA remained constant while the protein levels declined in response to *lin-4* recognition of the 3' UTR complementary sites (Wightman et al., 1993). Furthermore, the association of *lin-14* mRNA with polyribosomes appeared unchanged upon *lin-4*-mediated inhibition of protein expression (Olsen and Ambros, 1999). The generality of this type of gene regulation was supported by reports that the *lin-28* mRNA levels and polyribosome profile were not altered when *lin-4* blocked LIN-28 protein accumulation (Moss et al., 1997; Seggerson et al., 2002). However, the data shown in all of these reports were generated by RNase protection experiments, which do not demonstrate the existence of full-length, intact mRNA species.

Here, we use Northern analyses to compare the expression of full-length endogenous mRNA targets of miRNA regulation. By this technique, it is apparent that the relative levels of *lin-14* and *lin-28* mRNAs decrease upon the expression of *lin-4* miRNA. This change is dependent on wild-type *lin-4* activity and, at least in the case for *lin-14*, intact *lin-4* complementary sites in the 3' UTR. Although the relative contribution to overall gene expression by *lin-4*-mediated translational inhibition and mRNA degradation is yet to be determined, the *lin-4*-dependent decrease in *lin-14* mRNA levels is consistent with a significant role for mRNA degradation. The LIN-14 protein levels decrease ~10-fold from the L1 to the L2 stages of development (Olsen and Ambros, 1999; Wightman et al., 1993), but this reduction is only partially dependent on *lin-4* miRNA (Wightman et al., 1993). In fact, Wightman et al. demonstrated that the difference in LIN-14 protein downregulation between wild-type and *lin-4(e912)* mutants was 4-fold (Wightman et al., 1993), which is comparable to the fold decrease in *lin-14* mRNA levels reported here (Figure 5).

We cannot explain the discrepancy between our results and the previous studies of *lin-4*-mediated regulation. For our Northern experiments, we used probes

that covered regions analyzed in the RNase protection experiments. However, we did not detect stable degradation products corresponding to these genes that could account for the previous conclusion that the levels of *lin-14* and *lin-28* mRNAs are not significantly altered (Olsen and Ambros, 1999; Seggerson et al., 2002; Wightman et al., 1993). We note that Olsen and Ambros reported a 2.3-fold ( $\pm 0.96$ ) reduction in *lin-14* mRNA levels between the L1 and L2 stages of development (Olsen and Ambros, 1999), which is consistent with our RNA analyses that lead us to conclude that regulation by *lin-4* miRNA can affect target mRNA abundance.

Reporter-gene experiments showed that the *lin-41* 3' UTR containing intact *let-7* complementary sites mediates inhibition of gene expression in a *let-7*-dependent manner (Reinhart et al., 2000; Slack et al., 2000). The *lin-41* 3' UTR contains two similar *let-7* complementary sites clustered within 75 nt of each other (Reinhart et al., 2000; Slack et al., 2000). In *C. elegans*, reporter-gene regulation by *let-7* is exceptionally sensitive to alterations in this region (Vella et al., 2004a, 2004b), indicating that this element may act as a module for effective gene regulation in vivo. We find an overrepresentation of degradation products mapping to either side of the *lin-41* LCS region (Figure 4E). Presently, it is unclear if recognition of the LCS by *let-7* results in degradation via endonucleolytic cleavage that occurs near the LCS region or if decay products become stalled at the borders of sequences potentially protected by complexes bound to the *let-7*-LCS duplexes.

Although there does not appear to be a considerable delay between the decrease in *lin-14* mRNA and protein levels (Figure 5B), translational inhibition may precede destabilization of miRNA targets. In fact, miRNAs have been reported to copurify with polyribosome fractions (Kim et al., 2004; Nelson et al., 2004). Specific miRNA complexes may recognize cognate mRNA targets while they are being translated. Such interaction could result in translational inhibition followed by degradation of the targeted mRNA. The 5'-3' exonucleases typically recognize exposed 5' monophosphate-containing RNAs that can result from mRNA decapping or endonucleolytic cleavage (Parker and Song, 2004). The 3' RNA fragments that result from RNAi-directed endonucleolytic cleavage of target mRNAs are substrates for 5'-3' exonucleolytic degradation (Orban and Izaurralde, 2005; Souret et al., 2004). We find that apparently full-length, possibly decapped, *lin-41* mRNA and an ~500 nt degradation product accumulate upon depletion of 5'-3' exonuclease activity (Figure 4B), indicating that *let-7*-mediated regulation of its target also involves this degradation machinery. Recently, P bodies (also called cytoplasmic or GW bodies), which are cytoplasmic foci for localized mRNA degradation, have been implicated in the RNAi and miRNA pathways (Liu et al., 2005; Sen and Blau, 2005). The colocalization of 5'-3' exonucleases, decapping enzymes, argonaute proteins, and mRNA targets of miRNAs to P bodies in mammalian cells (Liu et al., 2005; Sen and Blau, 2005) supports the possibility that some miRNAs direct mRNA targets to these specialized centers for initiation and/or completion of mRNA degradation.

The primary outcome of a given miRNA-target in-

teraction may depend on mRNA levels and the type and context of the complementary site. Heterologous reporter systems in mammalian cells aimed at recapitulating the mechanism of miRNA regulation have supported the model that imperfect base-pairing between an ~22 nt RNA and target sites results in inhibition of protein synthesis without alteration of steady-state mRNA levels (Doench et al., 2003; Zeng et al., 2002, 2003). Additionally, in vitro studies using *Drosophila* extracts showed that *let-7* miRNA could only direct cleavage of target sites that supported complete duplex formation (Hutvagner and Zamore, 2002). In contrast, another in vitro study demonstrated that a minimal RISC purified from human cells could mediate cleavage at significantly mismatched target sites (Martinez and Tuschl, 2004).

There are several possible explanations for the apparent discrepancies between the results of previous reporter gene and in vitro studies and our present in vivo findings. Inhibition of translation or mRNA stability could depend on the precise nature of the miRNA:target interaction. Heterologous reporters containing artificial target sites may fail to elicit complete regulation but be sufficient to impair translation. Additionally, some transgene transcripts may be targeted for a different mode of regulation altogether (Grishok et al., 2005; Mello and Conte, 2004). The in vitro systems may not be competent for the mRNA destabilization activity. It is also possible that some bona fide miRNA:target interactions do inhibit translation without affecting mRNA levels, as indicated by the mammalian reporter systems (Doench et al., 2003; Zeng et al., 2002, 2003). In fact, the mechanism directed by a given miRNA could be dependent on multiple factors—entire target mRNA composition and expression levels, cell type or stage of development, and availability of the miRNA and its cofactors.

Recently, human miR16 was shown to be involved in mediating the instability of mRNAs containing AU-rich elements (Jing et al., 2005). This miRNA contains a sequence that is complementary to AU-rich elements found in unstable mRNAs and helps recruit tristetraprolin to such mRNAs, a factor that initiates mRNA decay by deadenylation (Lai et al., 1999). Accordingly, Jing et al. (2005) did not detect endocleaved mRNAs resulting from miR16 regulation. The degradation pathway mediated by *lin-4* and *let-7* miRNAs in *C. elegans* is likely to differ in some respects to that of miR16, as the *lin-4* and *let-7* miRNAs and their target complementary sites lack AU-rich sequences. The mechanism for downregulation of target mRNAs by *lin-4* and *let-7* miRNAs also appears to differ from that utilized by perfect complement miRNAs that direct “slicing” activity. RISC cleaves the mRNA strand of a perfectly base-paired miRNA or siRNA-target duplex at the phosphodiester bond between nucleotides 10 and 11 (Elbashir et al., 2001). At least in some cases, the 5′ cleavage product appears to contain stretches of nontemplated uridine addition after miRNA-directed cleavage (Shen and Goodman, 2004). We do not find evidence of cleavage in the middle of *let-7-lin-41* LCS partial duplexes or addition of nucleotides to the mRNA. We cannot rule out that such events happen, but the products are too short lived for experimental detection. Regulation of

*lin-41* mRNA by *let-7* apparently also differs from the well-studied RNA degradation pathways of nonsense-mediated decay and RNAi, as essential genes in these pathways, *smg-2*, *rde-1*, and *rde-4* are dispensable for *let-7*-directed destabilization of its target mRNA (Figure S3).

The identification and validation of plant miRNA targets has been robust because target sites are typically perfect or near-perfect complements of the miRNA, and duplex formation results in degradation of the target mRNA (Dugas and Bartel, 2004). The model that partial base-pairing between a miRNA and potential target affects protein but not mRNA levels has complicated experimental validation of the vast majority of animal miRNA target predictions (Lai, 2004). However, our observation that genetically defined targets of two different miRNAs in *C. elegans* are regulated at the level of mRNA accumulation demonstrates that direct analyses of mRNA levels may support miRNA target predictions, in at least some cases. Furthermore, several miRNAs in *Drosophila* have been predicted to target 3′UTR regulatory motifs that mediate downregulation of mRNA levels (Lai, 2002; Lai and Posakony, 1997; Lai et al., 1998, 2005), and introduction of exogenous miRNAs to mammalian cell culture can elicit downregulation of mRNA expression of genes containing partial complementary sites in their 3′UTRs (Lim et al., 2005). Thus, regulation of target mRNA levels may be a common output of miRNA:target interactions that form imperfect duplexes.

Cloning of the 3′ cleavage products resulting from near-perfect duplex formation between a miRNA and an mRNA sequence has been a powerful means of experimentally validating targets of miRNA action (Jones-Rhoades and Bartel, 2004; Kasschau et al., 2003; Llave et al., 2002; Mansfield et al., 2004; Tang et al., 2003; Yekta et al., 2004). Regardless of whether the *lin-41* 5′ and 3′ degradation products result from *let-7*-directed endonucleolytic cleavage or are decay intermediates that accumulate because of duplex formation with *let-7* miRNA complexes, they mark the position of a potential in vivo miRNA:target interaction. Cloning of such products for other mRNAs could reveal sites that may be recognized by specific miRNAs.

In conclusion, we have shown that regulation by miRNAs can result in destabilization of target mRNAs containing sites of imperfect complementarity in vivo. Thus, the output of at least some miRNAs is degradation of their specific target mRNAs. Our evidence that established targets of miRNAs are downregulated at the mRNA level provides a foundation for investigating the mechanism by which some miRNAs promote degradation of their targets. Furthermore, the demonstration that genetically defined targets of miRNA control in *C. elegans* are significantly downregulated at the mRNA level has broad implications for experimental validation of other miRNA:target predictions.

#### Experimental Procedures

##### Northern Analyses

Detection of small RNA species was carried out by polyacrylamide gel electrophoresis (PAGE) Northern methods (Pasquinelli et al., 2003) and Northern analyses of larger RNAs were performed by

separating total RNA in 1% agarose gels, using conditions previously detailed (Bracht et al., 2004). Quantification of RNA bands was done by PhosphorImager Imagequant analyses. The *eft-2* mRNA control was used for normalization, and graphs represent the averages and standard deviations for two or more experiments from independent samples.

#### RT-PCR Analyses

Standard RT-PCR analyses were performed as previously described (Bracht et al., 2004). RNA ligase-mediated rapid amplification of 5' and 3' cDNA cleavage products was accomplished with the GeneRacer Kit (Invitrogen), using a modified protocol whereby RNA ligation was performed on the RNA sample without prior phosphatase and 5' cap removal steps for the 3' products and with dephosphorylated total RNA and 5'-phosphorylated RNA oligonucleotide for 5' products. The qRT-PCR analyses were carried out by the TaqMan method and Applied Biosystems ABI Prism 7700 Sequence Detection System. The relative RNA amounts were calculated using the  $\Delta\Delta C_t$  method and normalized to *eft-2* control for endogenous mRNAs or GFP for transgenic mRNAs. Relative positions of the primers are indicated in Figure S1.

#### RNAi Experiments

Synchronized L1 hatchlings were cultured on RNAi plates seeded with bacteria containing vector control or plasmids expressing dsRNA corresponding to *xrn-1* or Y48B6A.3 5'-3' exonuclease genes (Kamath et al., 2003). RNA was isolated from worms collected at L2 and L4 stages and used for Northern and RT-PCR analyses.

#### Western Analyses

Lysates from synchronized worms were prepared by homogenization in buffer containing 10 mM HEPES (pH 7.0), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 1 mM DTT, 10% glycerol, and 1% protease inhibitor cocktail (Sigma). Protein samples were run on denaturing 4%–20% Novex Tris-Glycine gels (Invitrogen) and electroblotted to Immun-Blot PVDF membrane (Bio-Rad). Western analysis for LIN-14 protein was done as previously described (Reinhart and Ruvkun, 2001), using anti-LIN-14 C-terminal antibody (1:1000; Ruvkun and Giusto, 1989) and HRPO-conjugated anti-rabbit antibody (1:5000; Bio-Rad). Western analyses for RNA polymerase II were performed similarly using ChIP grade mouse monoclonal antibody (1:1000; 4H8 Abcam, Inc.).

#### Chromatin Immunoprecipitation (ChIP) Assays

ChIP experiments were based on previously described protocols (Chu et al., 2002; Sandoval et al., 2004) and the ChIP assay kit (Upstate Cell Signaling Solutions). Anti-RNA polymerase or control mouse serum precipitates were used in PCR to detect DNA corresponding to the coding regions of *lin-41* and *col-10* mRNA genes and the negative control 5.8S rRNA gene, which is transcribed by RNA polymerase I.

#### Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at <http://www.cell.com/cgi/content/full/122/4/553/DC1/>.

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