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A whole-genome RNAi screen for *C. elegans* miRNA pathway genes

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Summary

Background—miRNAs are an abundant class of small, endogenous regulatory RNAs. Although it is now appreciated that miRNAs are involved in a broad range of biological processes, relatively little is known about the actual mechanism by which miRNAs down-regulate target gene expression. An exploration of what protein co-factors are necessary for a miRNA to down-regulate a target gene should reveal more fully the molecular mechanisms by which miRNAs are processed, trafficked, and regulate their target genes.

Results—A weak allele of the *C. elegans* miRNA gene *let-7* was used as a sensitized genetic background for a whole-genome RNAi screen to detect miRNA pathway genes, and 213 candidate miRNA pathway genes were identified. About 2/3 of the 61 candidates with the strongest phenotype were validated through genetic tests examining the dependence of the *let-7* phenotype on target genes known to function in the *let-7* pathway. Biochemical tests for *let-7* miRNA production place the function of nearly all of these new miRNA pathway genes downstream of *let-7* expression and processing. By monitoring the down-regulation of the protein product of the *lin-14* mRNA, which is the target of the *lin-4* miRNA, we have identified 19 general miRNA pathway genes.

Conclusions— The 213 candidate miRNA pathway genes identified could act at steps that produce and traffic miRNAs or in downstream steps that detect miRNA::mRNA duplexes to regulate mRNA translation. The 19 validated general miRNA pathway genes are good candidates for genes that may define protein cofactors for sorting or targeting miRNA::mRNA duplexes, or recognizing the miRNA basepaired to the target mRNA to down-regulate translation.

Introduction

miRNAs are a class of small (~22 nt), non-coding RNAs that regulate gene expression through complementary base-pairing to target mRNAs, typically at sites located in the 3'UTR region [1]. In animals, discontinuous base-pairing between a miRNA and its target mRNA yields a secondary structure containing extensive loops and bulges that generally trigger potent inhibition of translation and/or a less robust degradation of the mRNA. Since the discovery of the first two miRNAs in *C. elegans*, hundreds of miRNAs have been identified in organisms from plants to flies to humans. miRNAs play roles in a wide variety of processes, including developmental events, programmed cell death, stem cell differentiation, neuronal specification

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and more [2]. Although our understanding of the basic biology of miRNA biogenesis and function has grown, there is still much we do not understand about the mechanisms by which miRNAs act to modulate message stability and translation.

As miRNAs play diverse roles in development, loss of general miRNA pathway factors would be expected to yield devastating phenotypes. Indeed, complete loss of known miRNA pathway factors, such as the ribonuclease III enzyme Dicer, cause early embryonic lethality [3–5]. Genetic screens aimed at uncovering miRNA pathway factors are hampered by the fact that the desired mutants would likely be lethal. This problem may be solved through the application of whole-genome RNAi screening in a background sensitized for defects in a specific miRNA pathway.

The *let-7* miRNA was the second miRNA discovered and the first demonstrated to be conserved in other organisms [6,7]. Genetic analysis of suppressors of *let-7* loss-of-function phenotypes in *C. elegans* has revealed several mRNAs targeted by *let-7*, including *lin-41*, which encodes a Ring finger protein and *hbl-1*, which encodes a Hunchback-like protein [8–10]. A null mutation in *let-7* causes retarded heterochronic phenotypes during larval development and lethality at the larval to adult transition when the worms are unable to properly form a vulva and consequently burst [7]. The crucial mRNA target of *let-7* with regard to the heterochronic bursting phenotype is *lin-41*; bursting is suppressed in *lin-41;let-7* double mutants [10]. Furthermore, down-regulation of *dcr-1* (the worm homolog of Dicer) by dsRNA injection, which impairs the processing of miRNAs, causes phenotypes strikingly similar to those of the *let-7* mutant, including vulval bursting [3]. Thus *let-7* is sensitive to changes in the activity of the general miRNA pathway.

A weak allele of *let-7*, *mg279*, shows slightly reduced levels of the mature *let-7* miRNA due to defects in processing of the primary transcript, but is viable and does not show the *let-7* null phenotype of lethality due to bursting [7,11]. We reasoned that this weak allele of *let-7* would provide a sensitized background in which to detect defects in the miRNA pathway, by producing a specific and scorable miRNA-associated phenotype rather than lethality. We carried out a whole-genome feeding RNAi screen and identified 213 gene inactivations that give rise to a bursting phenotype in a *let-7(mg279)* background. Examination of other phenotypes in the *let-7* temporal patterning pathway confirmed a role in the heterochronic pathway for many of the candidates. Northern blotting indicated that the new miRNA pathway genes function downstream of *let-7* expression and processing. Some of the gene inactivations also affected *lin-4* miRNA regulation of the *lin-14* transcript, implicating this subset of genes in processes generally required for miRNA function.

Results and Discussion

let-7(mg279) is sensitized for defects in the miRNA pathway

As in other organisms, the ribonuclease III enzyme DCR-1 is required for the processing of miRNA precursors to mature miRNAs in *C. elegans* [3,12,13]. Complete loss of DCR-1 function causes early embryonic lethality [3]. Probably because of partial inactivation of *dcr-1*, dsRNA targeting *dcr-1* causes no obvious phenotype in the first or second generation of feeding to wild-type *C. elegans* (Figure S1A). However, an accumulation of the *let-7* miRNA precursor can be detected by Northern blot, indicating that DCR-1 activity is decreased by *dcr-1* RNAi feeding(Figure 2 and [12]). We hypothesized that in the *let-7(mg279)* worms, where mature levels of *let-7* are already lower, *dcr-1* RNAi feeding might produce a genetic enhancement to the bursting *let-7* null phenotype. Indeed, *let-7(mg279)* worms fed *dcr-1* dsRNA show an increased incidence of bursting at the larval to adult transition as compared to *let-7(mg279)* worms fed a control RNAi clone or wild-type worms fed either *dcr-1* or control dsRNA (Figure S1A). Thus, *let-7(mg279)* worms are sensitized to detect subtle defects in the

miRNA pathway and provide a useful genetic background to perform an enhancer screen to identify new components of this pathway.

A genome-wide feeding RNAi screen uncovers 213 gene inactivations that enhance *eri-1 (mg366); let-7(mg279)*

The *eri-1(mg366)* mutation renders worms hypersensitive to RNAi[14]. The *eri-1(mg366); let-7(mg279)* double mutant exhibits significant bursting when fed *dcr-1* dsRNA (Figure S1A). The *eri-1(mg366)* single mutant also shows significant bursting when fed *dcr-1* RNAi, most likely due to the increased efficiency of gene knockdown, but the presence of the *let-7 (mg279)* allele increased the bursting response significantly (Figure S1A). We used this double mutant to perform a whole-genome feeding RNAi screen. The screen utilized the Ahringer feeding RNAi library supplemented with clones from the Vidal feeding RNAi library for a total of ~17,900 dsRNA clones, corresponding to 94% of the protein-coding genes in the *C. elegans* genome [15–17]. RNAi feeding was initiated with L1 larvae and their progeny were scored for bursting at the L4 to young adult transition. This two-generation screen gives RNA interference time to reach maximal gene inactivation. In addition, a cherry-picked library of approximately 2700 clones from the whole-genome library known to cause developmental arrest, lethality, and sterility in the first generation of an *eri-1* mutant strain [15] was screened by initiating feeding with L1 larvae and scoring for bursting at the larval to adult transition in the first generation of RNAi. Individual wells were scored on a 4 point scale, with 1 corresponding to ~1% burst, 2 corresponding to up to ~15% burst, 3 corresponding to up to ~50% burst and 4 corresponding to greater than ~50% burst. The bursting phenotype is highly penetrant in a *let-7* null mutant [10].

As an important set of controls, empty vector clones and *dcr-1* clones were added to the library and screened blindly. A total of 110 empty vector controls were scored and in each case caused no bursting, suggesting that the false positive rate of the screen should be very low (Figure S1B). Of the 112 *dcr-1* RNAi clones scored, the majority were assigned the strong bursting score of 3 or 4. However, 19 of the *dcr-1* clones were scored as generating lower levels of bursting and even, in 5 examples, no bursting at all. That *dcr-1* RNAi occasionally fails to induce bursting indicates that some real positives may be missed in the initial screen, possibly due to the variability of RNAi.

The initial screen identified 350 RNAi feeding clones, targeting 332 individual genes, that induce *let-7*-mutant-like bursting in the *eri-1(mg366); let-7(mg279)* background (Figure S1C). Upon re-testing in triplicate, 231 clones, targeting 213 individual genes, reproducibly generated this bursting phenotype (Table S1). It is interesting to note that the lethal/sterile sub-library was significantly enriched for clones that scored positive. Of these 213 genes, most are conserved across phylogeny: for example 172 of these genes are included among the KOG classification, which identifies genes having clear orthologs in plants, animals or fungi [18]. The animal and plant orthologs identified by the KOG classification of the miRNA pathway genes revealed in our screen are good candidates to mediate miRNA function in those phyla as well.

After eight individual trials, an average burst score was calculated for each positive RNAi clone. While all clones that reproducibly enhanced bursting may be considered positives, a cut-off was set at a score of 2 for initial more detailed analysis. In addition, due to the variability of RNAi, those clones that had an average score of less than 2, but at least two individual scores of 3 or 4 were also included. This strict positive category included 69 clones, targeting 61 genes (Table S1).

Genetic tests place many of the candidate genes in the *let-7* miRNA pathway

A trivial explanation for the bursting induced by the RNAi clones is that they target genes crucial for vulval development, but in pathways parallel to that of the *let-7* miRNA pathway. To address this we compared the bursting phenotype of a dsRNA clone on the *eri-1(mg366); let-7(mg279)* strain to that induced by feeding *eri-1(mg366)*. Given our hypothesis that the *let-7(mg279)* allele provides a sensitized background due to lower levels of mature *let-7*, we reasoned that those clones targeting genes required for *let-7* activity would be more likely to enhance the bursting phenotype in the presence of that allele. To measure the dependence of the bursting on *let-7(mg279)* we subtracted the bursting score of *eri-1(mg366)* from that of *eri-1(mg366); let-7(mg279)*. Bursting was considered dependent on *let-7(mg279)* if the difference was greater than 1.5. Inactivation of 20 of the 61 genes in the strict positive class showed a dependence on *let-7(mg279)* for the bursting phenotype (Figure 1B). These gene inactivations also failed to enhance the weak bursting phenotype of the unrelated multi-vulva mutant *lin-1(e1777)* (Figure S2), further arguing against general defects in vulval development.

dcr-1 RNAi by injection induces bursting even in the wild-type background [3], suggesting that *let-7* phenotypes can result from a down-regulation of the general miRNA pathway machinery even in the absence of a sensitized background. By this view, some of the RNAi clones that induce a bursting phenotype independently of the *let-7(mg279)* allele may still target genes in the miRNA pathway. To identify this class of gene targets, we asked if the induced bursting could be suppressed in the *lin-41(ma104)* background, which suppresses bursting of a *let-7* genetic mutant [7,10]. We used a similar approach to that described for examining *let-7 (mg279)* dependence, comparing the bursting induced in the *eri-1(mg366); let-7(mg279)* strain with a triple mutant, *lin-41(ma104); eri-1(mg366); let-7(mg279)*. The bursting induced by inactivation of 39 of the 61 strict positive genes was suppressed by *lin-41(ma104)* (Figure 1C). Taken together, these genetic tests pl ace 44 genes, targeted by 49 of the 69 strict positive RNAi clones, in the developmental pathway controlled by *let-7*.

Classes of candidate miRNA pathway genes identified

RNA binding/processing—Genes in one large class of candidate miRNA pathway genes encode proteins with known or predicted roles in RNA binding or processing. Included in this class is *dcr-1*, with its well-established role in miRNA processing [1]. Also included in this category is *imb-4*, which encodes the worm ortholog of exportin-1, an importin-β-like protein involved in nuclear trafficking. Although most similar to exportin-1, by BLAST *imb-4* also represents the closest worm homolog of exportin-5, which plays a role in the export of pre-miRNAs from the nucleus [1]. The known heterochronic pathway gene and miRNA target *lin-28* [19] also was identified in the screen. LIN-28 interacts with target mRNAs to regulate their translation efficiency and has been localized to stress-granules [20,21], which are related to P-bodies, and may be sites of miRNA regulation [21].

Several of the genes identified encode proteins predicted to act in mRNA processing. F32B6.3 encodes a protein with similarity to human HPRP18, which interacts with the U5 snRNP involved in splicing [22]. F37E3.1 encodes a conserved nuclear cap-binding protein, which has been found to interact with the m(7)G cap and also with translation initiation factors [23, 24]. A translation inititation factor itself, eif-3.D, was also identified, as was a DEAD-box RNA helicase, F01F1.7, which is predicted to interact with F37E3.1 [25]. Kiriakidou et al. identified a m(7)G cap-binding domain in human Ago2 that is required for translational inhibition and is conserved in *C. elegans alg-1* and *alg-2*, suggesting that the m(7)G cap plays a crucial role in miRNA regulation [26]. One of the strongest positives in the screen that was completely dependent on *let-7(mg279)* and suppressed by *lin-41(ma104)* was W04D2.6, which encodes a conserved protein with an RNP-1 RNA binding domain and a PWI domain. PWI

domains have been shown to bind both single-stranded and double-stranded nucleic acids [27], making this protein an intriguing candidate for a miRNA pathway component.

Cytoskeleton—A number of the candidate genes encode proteins that play a structural or regulatory role in the cytoskeleton. Y19D2B.1 and *tba-2* both encode α-tubulins and *mec-7* encodes a β-tubulin. *spd-1* encodes a microtubule interacting protein and *dyci-1*, which encodes an intermediate chain of the motor protein dynein, and *dnc-1*, which encodes the dynein interactor dynactin, also interact with microtubules. Two genes encoding members of the ADP-ribosylation factor family, *arf-1.2* and *arf-3*, were also identified. Members of this protein family play roles in controlling microtubule dynamics as well as intracellular trafficking [28]. *unc-59*, which encodes a septin, a filament-forming protein with known roles in cytokinesis [29], was also identified. Given the requirements for cell division and morphogenesis during vulval development, it would seem reasonable that these candidates may be required for cellular events downstream of the activity of *let-7*. Indeed, most of these gene inactivations cause bursting even in the absence of the *let-7(mg279)* allele (Figure 1B). However, this bursting is suppressed in the *lin-41(ma104)* background, raising the possibility that the connection between miRNAs and the cytoskeleton may be more tightly linked. In addition, inactivation of several of these cytoskeleton factors also abrogated miRNA down-regulation of *lin-14* mRNA translation (see below), arguing for a more general role in miRNA function. Evidence for a connection between small RNAs and the cytoskeleton does exist. In sea urchin eggs, Seawi, a homolog of the small-RNA factor Piwi, is a major component of the microtubule ribonucleoprotein complex that also includes ribosomes and mRNAs [30]. The discovery that RNAs interact with and play a regulatory role in mitotic spindle formation [31] provides a precedent for this potential miRNA/cytoskeleton regulatory connection. Although we have no direct evidence, it is tantalizing to imagine localized zones of miRNA regulation guided by association with the cytoskeleton.

Vulval induction signaling—Two signaling pathways that play well-characterized roles in induction of the vulva are the Wnt and the Ras-mediated pathways [32]. *pop-1*, which encodes the downstream transcription factor of the Wnt pathway was identified as a *let-7* enhancing gene inactivation, as was *bar-1*, which encodes the beta-catenin homolog that interacts with POP-1. We also identified *let-92*, which encodes a homolog of the catalytic subunit of protein phosphatase 2A that plays a positive role in Ras-mediated vulval induction [33]. *let-92* is thought to play a role downstream of *let-60*, the worm homolog of RAS and a possible target of the *let-7* family of miRNAs [34]. *lin-41(ma104)* suppresses the bursting caused by *let-92* gene inactivation, suggesting crosstalk between the pathways regulated by different *let-7* targets. These candidates represent a connection between the *let-7* miRNA pathway and the established vulval induction pathways.

Transcriptional regulation—Of most interest in this class is the Polycomb Group gene *sop-2*, which represses Hox gene expression and binds RNA [35]. *sop-2* was also identified in an RNAi screen for disruption of RNAi-mediated transcriptional gene silencing [36]. The identification of the *sop-2* inactivation as an enhancer of *let-7(mg279)* raises the possibility of a similar transcriptional silencing mechanism involving miRNAs. Interestingly, SOP-2 and another Hox gene regulator, SOR-1, are localized to a subnuclear compartment that may be where they function, for example, in epigenetic silencing [37]. *sor-1* was also detected as a strong *let-7* enhancer in our genome screen. The involvement of such polycomb-like factors in miRNA function suggests a possible transcriptional silencing aspect to miRNA function. Alternatively, these polycomb-like factors could positively regulate the transcription of miRNAs, though we do not see any change in *let-7* expression levels when these genes are inactivated (see below).

Several known or predicted transcription factors are also *let-7* enhancing gene inactivations, including *ceh-18*, *elt-1*, H20J04.3, Y53G8AR.9 and F13H6.1. Many miRNAs are expressed at higher levels in differentiated cells than tumor cells [38], suggesting that as cells differentiate during development, the expression of miRNAs is under significant transcriptional control. The transcription factors identified in our screen could be the direct regulators of miRNA expression that couple their activation to the differentiation of particular cell types during development. There is also evidence of post-transcriptional regulation of miRNA expression in tumors [39]; in these tumors, the cofactors for miRNA maturation or function could be under transcriptional control. Similarly, the transcription factors identified in our screen could control the expression of general miRNA pathway components, including other genes identified in this screen. In addition, *dpy-21*, which encodes a component of the dosage compensation complex was identified. It is also possible that miRNAs associate with these chromatin factors in the nucleus, in analogy to the siRNA regulation of heterochromatin formation in fungi.

Protein turnover—Inactivation of a proteasome subunit encoding gene, *pas-3*, or *skr-18*, which encodes a homolog of the SCF ubiquitin ligase component Skp-1, each induce *let-7 (mg279)*-dependent bursting. These gene identifications raise the intriguing possibility that active turnover of the proteins whose mRNAs are targeted by a miRNA may contribute to target down-regulation.

Protein traffickings—In addition to the two ADP-ribosylation factor family members mentioned above, *arf-2.1* and *arf-3*, two other genes encoding proteins involved in trafficking were uncovered. *cogc-4* encodes a conserved member of the oligomeric golgi complex which is required for proper golgi function and plays a role in vesicle trafficking [40]. *sft-4* encodes a conserved protein whose homologs are localized to transport vesicles [41]. With the exception of *arf-3*, for which the LIN-14 assay was not performed, each of these gene inactivations also inhibited LIN-14 down-regulation (see below), suggesting a general connection between protein trafficking and miRNA regulation.

Miscellaneous and unknown function—A number of candidate genes were identified that did not cluster into any obvious functional classification. These include Y50D7A.11, encoding another DEAD-box helicase that is predicted to act on DNA instead of RNA, two serine/threonine kinases encoded by B0285.1 and ZC581.1, a predicted GTPase encoded by B0207.6 and others. Five genes encoding proteins with unknown functions were also identified.

A common worry about all RNAi screens is specificity of the gene inactivation, most especially whether the dsRNA targets more than the intended mRNA. In general, RNAi screens in *C. elegans* have been validated to target the intended gene: for example dsRNAs targeting genes of known loss of function phenotypes from classical genetics induced just those phenotypes in most cases [17]. However, there are some indications that genes targeting some of the members of highly conserved protein families may inactivate more than the exactly homologous mRNA. For example, the *mec-7* dsRNA strongly enhances *let-7* and strongly affects LIN-14 down-regulation, but a *mec-7* null mutation is viable and is only known to be mechanosensory defective, and the *mec-7* tubulin is only expressed in mechanosensory neurons [42]. Blastn analysis of *mec-7* shows that there are regions of 20 to 30 nt conserved between beta tubulin paralogs that may be broadly targeted by these dsRNAs. However, most of the hits from the RNAi screen do not target members of conserved families of paralogs. In addition, several of the genes identified (*ceh-18*, *sop-2*, *dyci-1* and Y53G8AR.9) were targeted by multiple non-overlapping RNAi clones, all of which scored as positives, arguing against off-target effects of specific clones.

Known miRNA pathway components—The screen identified the known miRNA pathway components *dcr-1* and *imb-4*, the closest homolog of Exportin-5. This confirms the

utility of the sensitized *eri-1(mg366);let-7(mg279)* background for screening. There are, however, known miRNA pathway components that were not identified in the screen, either because they induced a more severe lethal phenotype or because they did not induce any detectable phenotype in the strain screened. The Argonaute family member *alg-1* has been shown to play a role in miRNA function in *C. elegans* and was not identified in our screen. More direct tests using known miRNA pathway genes showed that, in the *eri-1(mg366)* enhanced RNAi background, *alg-1* RNAi causes a larval lethal phenotype in the parental generation (data not shown); these arrested animals could not be scored for the*let-7* phenotype at the larval/adult transition. Two other known miRNA pathway components, *drsh-1* and *pash-1*, which mediate the processing of pri-miRNAs, were also not identified in the screen, but not due to earlier lethality. Interestingly, feeding RNAi targeting these genes does lead to bursting in some genetic backgrounds. We found that *let-7(mg279)* worms carrying a GFP transgene expressed in the hypodermis were enhanced to bursting upon feeding of dsRNA targeting *drsh-1* or *pash-1*, although feeding to *let-7(mg279)* or the GFP transgene strains alone did not induce bursting (data not shown). It is unclear why this transgene sensitizes *let-7 (mg279)* to *drsh-1 and pash-1* knock-down while adding *eri-1(mg366)* in the genetic background did not. These observations illustrate that while our sensitized RNAi screen approach will identify many miRNA factors, some bona fide miRNA pathway components may be missed.

Many of the candidate miRNA pathway gene inactivations induce phenotypes consistent with defects in the miRNA function

For the 44 candidate genes, we carried out further secondary tests to confirm a role in the *let-7* miRNA pathway. *let-7* has a well-characterized role in the heterochronic developmental pathway in *C. elegans* that determines the timing of specific cellular events during development. One of the final readouts of the heterochronic pathway is the expression of the cuticle collagen gene *col-19* in the hypodermis and seam of adults. The *let-7(mg279)* allele causes modest defects in the expression of a *col-19::GFP* fusion gene, with 11% of worms showing either no GFP signal or GFP in the seam, but not in the hypodermis (Figure 1D and [43]). dsRNA treatment led to a statistically significant increase in worms displaying disrupted *col-19::GFP* expression for 38 of the 42 candidate genes for which the assay could be performed (Figure 1D).

Another assay for the mis-coordination of developmental events is the presence of defects in adult alae, the small ridges that run along both sides of the adult. Loss or knockdown of many heterochronic pathway gene products and also of known miRNA pathway components causes defects in these alae, including the presence of gaps in the ridges [3,7,10,12]. The candidate RNAi clones were fed to *eri-1(mg366)* worms and the state of the adult alae were assessed visually for any gaps. Inactivation of 19 of the 44 genes caused significant defects in the continuity of adult alae (Figure 1E). It is significant that each of these assays examine heterochronic phenotypes in a tissue that is distinct from the vulval cells scored in the primary screen.

Most of the genes act downstream of *let-7* biogenesis

To determine if any of the targeted genes are required for *let-7* biogenesis, Northern blots with *let-7* probes were performed on samples from young adult animals, when *let-7* is normally highly expressed, after inactivation of each of the candidate miRNA pathway genes. The fact that the RNAi clones examined produce a bursting phenotype in the *eri-1(mg366); let-7 (mg279)* background presents a hurdle for collecting staged young adult animals on which to perform *let-7* Northerns. We knew that feeding RNAi targeting *dcr-1* to wild-type worms leads to a noticeable increase in *let-7* precursor accumulation, although there is no bursting phenotype associated with this molecular phenotype. Given this observation, we expected gene

inactivations affecting *let-7* miRNA processing may produce a molecular phenotype in the absence of bursting in the wild-type background. *dcr-1* RNAi demonstrated a clear, although incomplete, defect in processing of the *let-7* precursor to the mature form in young adults, as expected (Figure 2 and Figure S3). In contrast, no other dsRNA treatment induced a dramatic increase in precursor or decrease in mature *let-7* (Figure 2 and Figure S3). A subset of RNAi clones were also fed to *lin-41(ma104);eri-1(mg366);let-7(mg279)* worms to determine if miRNA processing defects could be detected in this genetic background, but again no defects in *let-7* maturation were observed (data not shown). These data suggest that these miRNA debilitating gene inactivations abrogate function downstream of the expression and processing of *let-7*.

Many of the miRNA pathway genes are necessary for the down-regulation of target mRNA translation

To classify the functions of candidate genes as either acting generally in the miRNA pathway or specifically to the *let-7* pathway, we assayed whether these gene inactivations also interfere with the *lin-4* miRNA pathway. *lin-4* was the first miRNA identified and it is well established that *lin-4* down-regulates *lin-14* mRNA translation during larval development [44,45]. Western blots of LIN-14 protein comparing levels in L1 and L2 larval stage worms show a clear down-regulation of protein in wild-type (Figure 3). We asked if RNAi inactivation of the miRNA pathway candidate genes abrogates this down-regulation of LIN-14 protein levels. LIN-14 runs as a doublet at ~67 kD. During normal development, the top ~67 kD band disappears first, followed by the lower band. For many of the *let-7*-enhancing gene inactivations, the down-regulation of LIN-14 at late stages was not as pronounced as in wild-type. For a significant subset of the miRNA pathway gene inactivations, in addition to the slower disappearance of the ~67 kD LIN-14 bands, a new LIN-14 band appears at ~75 kD. This ~75 kD band appears transiently in a wild-type time course of LIN-14 down-regulation (data not shown), suggesting that it is a short-lived intermediate in the wild-type pathway that becomes more stable when steps in the miRNA pathway are inactivated. The appearance of the ~75 kD band coincides in wild-type with loss of the upper band of the ~67 kD doublet, raising the possibility of a protein modification, such as sumoylation or ubiquination, during turnover (data not shown). Inactivation of 19 of 34 genes in the *eri-1(mg366)* background caused an increase in LIN-14 abundance at ~67 kD or ~75 kD as compared to comparably staged worms fed vector alone controls (Figure 1F, Figure 3). This is consistent with a role for these genes in the general miRNA pathway upstream of miRNA-mediated down-regulation of protein synthesis. The enhanced *let-7* miRNA phenotype resulting from knockdown of these genes in the whole-genome screen cannot be explained by a decrement in *lin-4* activity only, as the *lin-4 (e912);let-7(mg279)* double mutant does not show enhanced bursting (data not shown). In order to collect L1 and L2 larval samples that had been exposed to RNAi for the lethal/sterile clones, we started RNAi treatment in the first generation as late L4s. Even this late treatment led to lethality or sterility for many of the sterile/lethal clones, so only a subset of these clones was tested. With this caveat in mind, it is notable that almost all of the sterile/lethal RNAi clones for which we were able to perform the assay showed altered LIN-14 levels.

Taken together with the *let-7* Northern blot data, these LIN-14 results suggest that a major subset of the genes identified in our screen function between the point of miRNA biogenesis and the point of target mRNA down-regulation. Given how little is known about how miRNAs actually induce down-regulation of target mRNA translation and/or stability, the genes identified in this screen are promising leads towards discerning the molecular mechanisms by which miRNAs down-regulate their targets. In this set are several genes from the RNA binding/processing class, including the RNA binding W04D2.6, the nuclear cap-binding F37E3.1 and the predicted U5 snRNP interactor F32B6.3. It may be that these factors facilitate the interaction of the target mRNA with its corresponding miRNA or detect that interaction to in-turn recruit

the mRNA::miRNA complex to P bodies and down-regulate translation. It is also notable that all of the sterile/lethal cytoskeleton factors for which we were able to perform LIN-14 Westerns abrogated LIN-14 down-regulation, including the ARF family member *arf-1.2*, the dynactin *dnc-1* and both α-(*tba-2*) and β-(*mec-7*) tubulins. This suggests a more general role for the cytoskeleton in miRNA regulation. Perhaps subcellular sites of miRNA regulation, such as the P-bodies, stress granules, or other unknown sites, are organized by the cytoskeleton.

The miRNA pathway screen has identified genes distinct from those identified in a screen for RNAi factors

DCR-1 is an example of a protein that functions in both the miRNA and RNAi pathways [3, 12,13]. Kim et al. performed a whole-genome RNAi screen designed to identify genes required for RNAi. They identified only three genes *(dcr-1, pop-1* and *kin-10*) from their list of 90 candidates that when inactivated caused significant enhancement of *let-7(mg279)*, suggestive of a role in the miRNA pathway as well [15]. Consistent with this, only two of our 44 confirmed *let-7* pathway genes (*dcr-1* and *pop-1*) appear on the RNAi candidate list. It appears that, by these assays, the RNAi and miRNA pathways are largely molecularly distinct.

Experimental Procedures

Strains—Standard *C. elegans* cultivation techniques were used for all worm handling. Strains and mutant alleles used in this study: wild-type Bristol N2, *let-7(mg279)X*, *eri-1(mg366)IV*, *eri-1(mg366)IV; let-7(mg279), lin-41(ma104)I; eri-1(mg366)IV; let-7(mg279)X, veIs13 [col-19::GFP;rol-6(su1006)]V; let-7(mg279)X, Is: [col-10p::lac-Z-lin-41 3'UTR]* (integrated transgene strain kindly provided by the Plasterk lab).

RNAi screen for enhancement to bursting—Handling of the Ahringer RNAi library supplemented with the unique clones from the Vidal RNAi library and the cherry-picked sterile/lethal sub-library was done largely as previously described [15–17]. Synchronized L1 worms of the eri-1(mg366);let-7(mg279) genotype were placed onto 6-well RNAi plates; ~6 (for second generation screening) or ~100 (for parental generation screening). These RNAi plates were then kept at 20 °C and either scored for bursting 3 days later (for parental generation screening) or shifted to 25 °C 3 days later and then scored for bursting 2 days after that (for second generation screening). All scoring was done using a Nikon SMZ645 dissection microscope. Subsequent re-testing and bursting assays in other genetic backgrounds were performed as above.

RNAi controls—RNAi clones targeting *dcr-1* and the empty vector feeding clone pPD129.36 [46] were used as positive and negative controls, respectively. For the initial screening, these clones were added to approximately half of the 96-well O/N cultures in the place of clones known to give no growth. They were then scored blindly.

***col-19::GFP* assays—**Gravid adult *let-7(mg279)* worms carrying an integrated transgene expressing GFP under the *col-19* promoter and fed RNAi as above were examined for their GFP expression pattern with a fluorescence dissection microscope. Worms were scored as either have normal expression (in the hypodermis and seam) or disrupted expression (absent or in the seam alone). Chi squared analysis was performed to determine significance, with the cut-off at a p-value of 0.05.

Alae assays—Young adult *eri-1(mg366)* worms fed RNAi as above were mounted for imaging and the alae examined for gaps with DIC microscopy using a 40X lens. It should be noted that it was not possible to score alae in worms that had burst, so for those gene inactivations that induce some bursting in the *eri-1(mg366)* background, only worms that had survived past the larval/adult transition were scored. This could lead to an underestimate of

the impact of the gene inactivation on alae formation. Chi squared analysis was performed as for the col-19::GFP assays.

***let-7* Northern blots—**RNAi feeding in a worm strain carrying a reporter transgene were performed largely as for the bursting screen, with a few exceptions. In order to prepare RNA from synchronized worms in the second generation an egg prep was performed on the gravid adults from the RNAi-fed parental generation and the resulting synchronized L1s were then plated onto the same RNAi clone and grown to adulthood. Total RNA preparation and Northern blots for the *let-7* precursor and mature miRNA were performed as previously described [7], with the exception that Invitrogen 15% TBE-Urea gels were used and ULTRAHyb (Ambion) was used for the hybridization solution. 20 μg total RNA was loaded into each lane, except for the last 7 lanes for blot H, where 10 μg was loaded. Blots were reprobed for the U6 snRNA as a loading control.

LIN-14 Western blots—RNAi feeding was performed as described for the *let-7* Northern blots for RNAi clones scored in the second generation. For the sterile/lethal RNAi clones, worms were initially fed on the empty vector control and then transferred to RNAi plates as L4s before subsequent egg prepping of gravid adults. In both cases, a fraction of the synchronized L1s were flash-frozen in liquid nitrogen and others were placed onto the appropriate RNAi clone and fed for 24 hours at 20 °C before flash-freezing as L2s. Before L2 harvesting, worms were visually inspected to verify they were all at the same developmental stage. Worm lysate preparation and LIN-14 Western blotting was performed as previously described [47]. 50 μg of total protein (as assayed by Bio-Rad DC protein assay) was loaded into each lane of an Invitrogen NuPAGE 4–12% Bis-Tris gel. Blots were re-probed with an actin antibody (MP Biomedicals 691001) as a loading control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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	sequence name	locus	burst score[A]	*let-7(mg279)*-dependent[B]	*lin-41(ma104)*-suppressed[C]	disrupted *col-19::GFP*[D]	alae gaps[E]	LIN-14 perdurance[F]	gene class	brief description
	vector		0.0			11.0 (6.9)	0.7 (1.1)		control	empty dsRNA expression vector
viable clones	K12H4.8	*dcr-1*	2.5			63.4 (8.8)	58.3 (9.8)		RNA	RNAse III enzyme, required for RNAi and miRNAs
	Y59A8B.1	*dpy-21*	2.0			53.0 (18.4)	0.0		RNA	Dosage Compensation Complex component
	F32B6.3		2.5			99.0 (1.9)	0.0		RNA	human HPRP18 protein-like
	F02E9.2	*lin-28*	2.2			15.8 (5.4)	0.0		RNA	heterochronic pathway component. Target of lin-4
	W04D2.6		3.4			92.5 (13.8)	0.0		RNA	RNA recognition motif. (aka RRM, RBD, or RNP domain)
	Y34D9A.4	*spd-1*	2.5			53.9 (11.7)	81.3 (5.7)		cyto	conserved microtubule interacting protein
	W09C5.2	*unc-59*	2.5			66.7 (12.3)	85.4 (1.2)		cyto	septin
	ZC64.3	*ceh-18*	2.6			92.2 (6.8)	0.0		transcription	homeobox protein
	F13H6.1		2.1			30.0 (4.5)	ND		transcription	zinc-finger protein
	H20J04.3		1.9			94.4 (9.0)	84.6 (9.3)		transcription	predicted txn factor
	C50E10.4	*sop-2*	3.0			53.5 (2.0)	ND		transcription	polycomb group protein. RNA binding
	Y53G8AR.9		2.6			94.4 (8.1)	0.0		transcription	zinc-finger protein
	F56B3.4	*skr-18*	2.2			42.8 (7.5)	38.9 (17.3)		prot. turnover	skp-1 homolog. SCF component
	C54H2.5	*sft-4*	2.7			31.1 (1.3)	ND		trafficking	conserved protein. dilysine motif.
	B0207.6		3.0			89.8 (0.4)	33.3 (1.8)		misc.	predicted GTP-ase
	F59E10.1	*orc-2*	2.1			45.6 (10.1)	69.4 (7.5)		misc.	origin recognition complex, second largest subunit ORC2
	Y55F3AM.4		2.2			75.5 (8.3)	27.9 (9.9)		misc.	autophagy protein
	C15C6.1		3.2			40.6 (6.2)	88.6 (7.3)		unknown	unknown function
	Y39A1A.13		2.3			47.6 (5.8)	46.7 (10.1)		unknown	unknown function
	Y87G2A.1		2.2			53.0 (18.4)	37.9 (0.3)		unknown	unknown function
sterile/lethal clones	R08D7.3	*eif-3.D*	1.8			3.8 (3.5)	4.0		RNA	translation initiation factor 3, subunit D
	F01F1.7		2.4			22.2 (11.9)	8.0	ND	RNA	predicted DEAD-box RNA helicase
	F37E3.1		2.5			91.3 (9.9)	62.5 (12.9)		RNA	CAP-binding protein
	ZK742.1	*imb-4*	2.4			100 (0)	56.8 (20.1)		RNA	importin beta, nuclear transport factor
	B0336.2	*arf-1.2*	1.9			58.0 (8.4)	ND		cyto/trafficking	ARF family member
	F57H12.1	*arf-3*	2.5			89.2 (5.2)	ND	ND	cyto/trafficking	ARF family member
	C17H12.1	*dyci-1*	2.9			76.2 (10.2)	7.7	ND	cyto	dynein intermediate chain
	ZK593.5	*dnc-1*	2.6			81.9 (11.5)	0.0		cyto	dynactin
	ZK154.3	*mec-7*	2.6			26.6 (2.9)	9.8 (45.5)		cyto	beta tubulin
	C47B2.3	*tba-2*	2.3			77.8 (16.5)	0.0	ND	cyto	alpha tubulin
	Y19D2B.1		3.1			83.5 (2.0)	0.0		cyto	alpha tubulin
	C54D1.6	*bar-1*	3.7			39.7 (5.2)	0.0	ND	vulval	beta-catenin. Wnt signaling component
	F38H4.9	*let-92*	2.5			ND	72.0 (17.5)	ND	vulval	serine/threonine protein phosphatase
	W10C8.2	*pop-1*	2.4			28.7 (2.8)	53.1 (2.1)	ND	vulval	wnt signaling pathway component
	W09C2.1	*elt-1*	3.3			6.5 (4.7)	58.1 (11.3)	ND	transcription	elongation factor ELT
	Y110A7A.14	*pas-3*	3.0			100 (0)	7.7	ND	prot. turnover	endopeptidase. proteasome subunit
	Y51H7C.6	*cogc-4*	2.3			31.6 (5.8)	9.1		trafficking	conserved component of Golgi complex
	B0285.1		2.0			3.7 (1.7)	0.0		misc.	serine/threonine kinase (CDC2/CDKX subfamily)
	F25B4.6		2.9			ND	0.0		misc.	hydroxymethlglutaryl-CoA synthase
	Y50D7A.11		2.4			93.5 (7.6)	46.5 (18.8)		misc.	predicted DEAD-box DNA helicase
	ZC581.1		2.5			90.8 (5.6)	ND		misc.	ser/thr-protein kinase
	F48C1.4		2.0			58.5 (12.7)	40 (21.9)		unknown	unknown function
	Y110A7A.11		2.8			100 (0)	4.0	ND	unknown	conserved protein of unknown function
	ZK1236.3		3.7			9.3 (8.2)	0.0		unknown	unknown function

Figure 1. *let-7* miRNA pathway candidate genes

A. The average burst score from all trials (7–11 trials for each).

B. Gene inactivations for which bursting depends on the presence of the *let-7(mg279)* allele. Positives are defined as a difference of at least 1.5 in average bursting score over 3 trials comparing *eri-1(mg366); let-7(mg279)* to *eri-1(mg366).*

C. Gene inactivations where bursting is suppressed by *lin-41(ma104).* Positives which are shaded are defined as a difference of at least 1.5 in average bursting score over 3 trials comparing *eri-1(mg366); let-7(mg279)* to *lin-41(ma104); eri-1(mg366); let-7(mg279).*

D. Percentage of *col-19::GFP;let-7(mg279)* worms with disrupted patterns of expression. Standard deviation of three trials is shown in parentheses. Shading indicates a p value ≤ 0.05 determined by Chi square test comparing RNAi feeding to empty vector control.

E. Percentage of *eri-1(mg366)* worms with gaps in adult alae after RNAi feeding. Standard deviation of three trials is shown in parentheses, when appropriate. Shading indicates a p value ≤ 0.05 determined by Chi square test comparing RNAi feeding to empty vector control.
F. Shading indicates a perdurance of LIN-14 protein in L2 worms as determined by a comparison on a LIN-14 Western blot to the empty vector-fed control (see Figure 3).

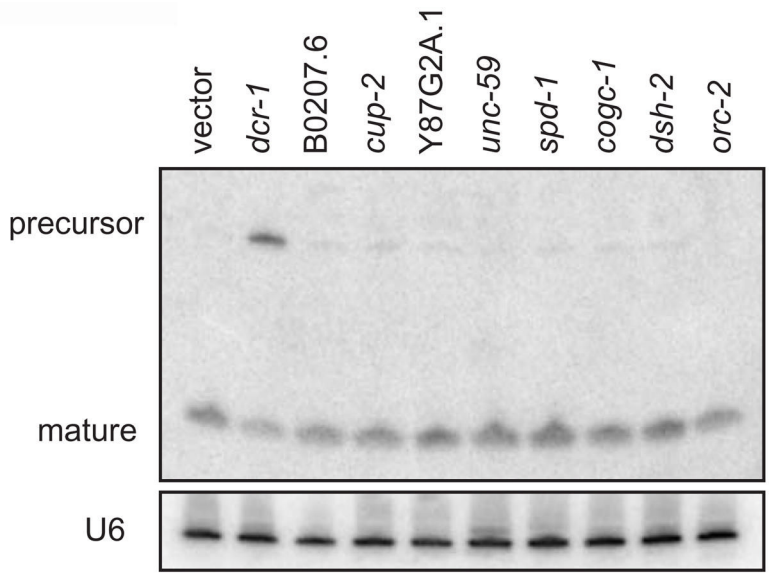

Figure 2. *let-7* Northern blot

Total RNA from young adult worms raised for two generations on feeding RNAi probed for *let-7*. *dcr-1* (RNAi) results in an accumulation of precursor and loss of mature *let-7* miRNA. Other RNAi treatments do not affect *let-7* processing or expression.

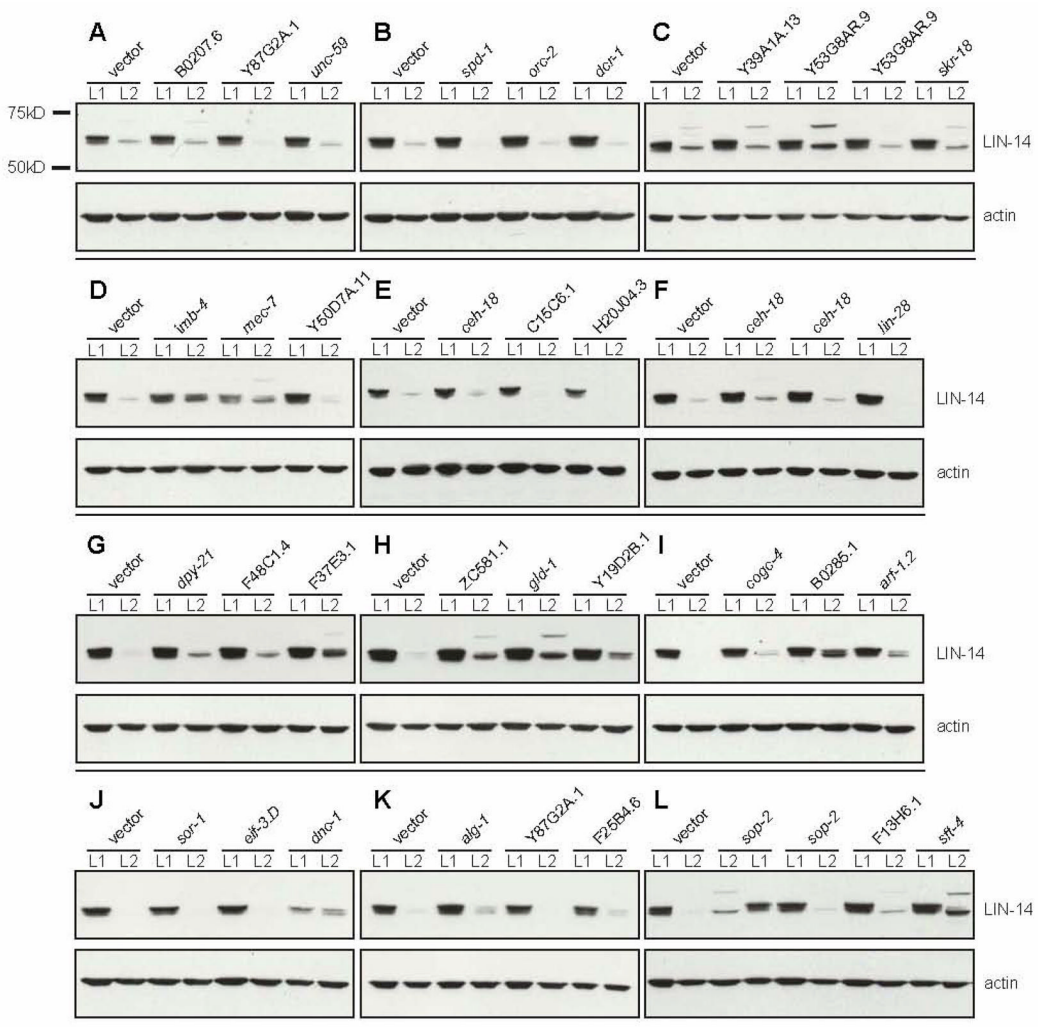

Figure 3. LIN-14 Western blots

Protein samples from synchronized L1 and L2 (24 hours at 20 °C) worms are shown for each RNAi treatment. LIN-14 levels drop dramatically from L1 to L2 when fed empty vector control. Due to variations in LIN-14 intensity between blots all comparisons were made to the empty vector control lanes for each blot to determine if LIN-14 down-regulation was affected.