Functional Identification of MicroRNA-Centered Complexes in *C. elegans*.

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Abstract

microRNAs (miRNAs) are crucial for normal development and physiology. To identify factors that might coordinate with miRNAs to regulate gene expression, we used 2'-O methylated oligonucleotides to precipitate *Caenorhabditis elegans* let-7, miR-58, and miR-2 miRNAs and the associated proteins. A total of 211 proteins were identified through mass-spectrometry analysis of miRNA co-precipitates, which included previously identified interactors of key miRNA pathway components. Gene ontology analysis of the identified interactors revealed an enrichment for RNA binding proteins, suggesting that we captured proteins that may be involved in mRNA lifecycle. To determine which miRNA interactors are important for miRNA activity, we used RNAi to deplete putative miRNA co-factors in animals with compromised miRNA activity and looked for alterations of the miRNA mutant phenotypes. Depletion of 25 of 39 tested genes modified the miRNA mutant phenotypes in three sensitized backgrounds. Modulators of miRNA phenotypes ranged from RNA binding proteins RBD-1 and CEY-1 to metabolic factors such as DLST-1 and ECH-5, among others. The observed functional interactions suggest widespread coordination of these proteins with miRNAs to ultimately regulate gene expression. This study provides a foundation for future investigations aimed at deciphering the molecular mechanisms of miRNA-mediated gene regulation.

Introduction

Developmental and physiological processes require precise spatio-temporal regulation of gene expression. One post-transcriptional gene regulatory mechanism is directed by a class of small non-coding RNAs called microRNAs (miRNAs). miRNAs regulate a wide range of developmental and cellular processes, with dysregulated miRNA activity prevalent in diseases^{1,2}. To exert their regulatory roles, miRNAs are loaded into Argonaute (AGO) proteins to form a miRNA Induced Silencing Complex (miRISC), which ultimately associates with an effector protein GW182. miRISC binding to the target mRNA via partial sequence complementarity between a miRNA and 3' UTR of mRNA triggers a series of gene silencing mechanisms including translation inhibition, decapping, and mRNA decay^{3,4}.

miRNAs are produced by a complex biogenesis process which involves enzymatic processing of miRNA intermediates in the nucleus and cytoplasm. Primary miRNAs are first cleaved by the Microprocessor complex (Drosha and DGCR8) to form pre-miRNAs^{5,6}. After export from nucleus into cytoplasm, pre-miRNAs are further processed by Dicer to generate a miRNA duplex^{7,8}. The miRNA duplex bound by Argonaute is then unwound, the guide miRNA strand retained to form the mature miRISC, and the passenger strand released and degraded^{9,10}. Each of the steps in miRNA biogenesis process can be regulated by RNA binding and other auxiliary factors thereby modulating the final gene-regulatory impact of miRNAs. These factors could bind miRNA intermediates or miRISC protein components to affect miRNA activity. For example, several RNA binding proteins including RBFOX3 and HnRNP A1 have been identified to bind to the hairpin structures of primary miRNAs and modulate their processing^{11,12}. Other proteins, such as NHL-2 and CGH-1, associate with ALG-1 and AIN-1 to promote mRNA targeting¹³. RNA binding proteins Staufen¹⁴ and HuR^{15,16} indirectly affect miRNA-mediated gene silencing by competing

for binding of the 3'UTRs of target mRNAs. Characterizing miRISC-associated protein complexes followed by functional analyses and mechanistic studies has high potential to identify additional mechanisms by which miRNA activity may be regulated.

Identifying proteins that associate with Argonaute proteins or miRNAs has been a productive approach to begin unravel the mechanisms by which aspects of miRNA biogenesis and activity are regulated^{17,18,19,20}. In human cells, investigation of proteomic profiles of AGO complexes led to identification of common protein interactors of all four AGO proteins, which included heat shock proteins, helicases, and components of translational machinery¹⁷. Some proteins identified in this study, including Hsc70/Hsp90 chaperone machinery, was later characterized for their roles in RISC loading of small RNA duplexes²¹. In mice, exploration of Dicer dependent and independent interactions of Ago2¹⁸ identified proteins that participate in miRISC-mediated decapping²², among other mechanisms. However, while proteomic approaches have characterized miRNA-associated complexes, it has been challenging to identify which of these co-factors are functionally important for miRNA activity, especially in tissue culture. In contrast, functional assays that quantitatively assess miRNA activity are available in model organisms such as *C. elegans*.

To identify miRNA/miRISC auxiliary cofactors important for miRNA gene regulatory activity, we took a functional proteomics approach. Specifically, we used 2'-O-methylated biotinylated oligonucleotides to pull down three miRNAs of interest (*let-7*, *miR-58*, and *miR-2*) and subjected the the associated protein complexes to proteomic analysis. Comparative analysis of miRNA pulldown and ALG-1 immunoprecipitation precipitates¹⁹ identified high confidence interactors common to all four datasets. In addition, we identified a unique set of interactors in each miRNA pulldown dataset. To assess whether the co-precipitated proteins are functionally important for miRNA activity, we performed RNAi knockdown of genes encoding for the putative physical interactors in multiple miRNA sensitized genetic backgrounds. Of the 39 interactors tested, depletion of 25 factors modified miRNA reduction of function phenotypes in one or more assays. Overall, we demonstrate that capturing physical interactors of miRNA machinery followed by *in vivo* functional assays is an efficient approach to identify novel players in miRNA-mediated gene regulation. While further mechanistic characterizations are necessary to determine the extent of the physical and functional interactions, this study identifies a functional requirement for a subset of potential ALG-1 and miRNA co-factors.

Methods

C. elegans maintenance, strains, and RNAi

All *C. elegans* strains were maintained on NGM and fed with *E. coli* OP50. Strains were maintained at 20°C unless otherwise noted. RNAi knockdown was performed by feeding as previously described²³.

The following strains were used in this study: N2 (wild type), MT7626 (*let-7(n2853)*), HW1113 [*Pdpy-30::GFP(PEST)-H2B::lin-41 3' UTR (xeSi78)*; *Pdpy-30::mCherry::H2B::artificial 30 UTR (xeSi36)*], HW1114

[*Pdpy-30::GFP (PEST)-H2B::lin-41 3' UTR (xeSi78)*; *Pdpy-30::mCherry::H2B::artificial 3' UTR (xeSi36)*, *let-7(n2853)*], VT1367 (*col-19::gfp (mals105)*), VT1296 (*mir-48 mir-241(nDf51) col-19::gfp (mals105)*), BW1932 [*hbl-1p::gfp::NLS::hbl-1 3' UTR (ctlS39)*] and UY458 (*mir-48 mir-241(nDf51)*; *hbl-1p::gfp::NLS::hbl-1 3' UTR (ctlS39)*), OH812 (*otls114 [Plim-6-gfp + rol-6(su1006)]*), OH3646 (*lsy-6(ot150)*; *otls114 [Plim-6-gfp + rol-6(su1006)]*), PS3662 (*syls63[cog-1::gfp + unc-119(+)]*), OH7310 (*otls193 [cog-1p::lsy-6 + rol-6(su1006)] syls63[cog-1::gfp + unc-119(+)]*).

2'O-methyl Oligo Pulldowns and Mass spec analysis

Whole worm extracts²⁴ and 2'O-methyl oligo pulldowns²⁵ were performed as previously described. For mass spectrometry, each sample contained 20 mg of total protein. miRNA pulldowns were performed in two biological replicates using 2'O-methylated oligos with perfect complementation to miR-58, let-7, and miR-2, and scrambled oligo control (IDT). Sequences of the 2'O-methylated, biotinylated oligonucleotides are as follows: miR-58 oligo (5'-CAUCAUUGCCGUACUGAACGAUCUCAAGUC-3'), miR-2 oligo (5'-AUUCAGCACAUCAAAGCUGGCUGUGAUUAUCCA-3'), let-7 oligo (5'-UCUUCACUAUACAACCUACUACCUCAACCUU-3'), and scrambled oligo (5'-CAUCACGUACGCGGAUACUUCGAAAUGUC-3').

Mass spectrometric analysis of pulldown factors was performed as previously described¹⁹. Briefly, DTASelect²⁶ was used to filter the proteins identified by applying a criterion that required proteins to have at least two unique peptides with total spectral intensities greater or equal to four. To determine enrichment of protein association in a miRNA pulldown, the Normalized Spectral Abundance Factor (NSAF) values in miRNA pulldown were divided by that in control pulldown. NSAF value of zero in control was replaced by 1. Proteins with the experimental/control ratio of ≥ 4 in all replicates were considered putative physical interactors.

GO term and Network analysis

Gene ontology analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID)²⁷. Factors that had a fold change ≥ 4 in both replicates of the miRNA pulldowns were used for this analysis. For comparison, we included factors identified in at least two replicates of ALG-1 IP for GO term analysis (as previously described¹⁹). Protein domain information was retrieved from STRING²⁸. Network analysis was performed on the top 40 most enriched factors using STRING²⁸ after excluding ribosomal proteins.

Functional assays

let-7(n2853) vulval bursting assay

Vulval bursting assay was performed as previously described²⁹. Briefly, *let-7(n2853)* and N2 worms were grown and maintained at 15°C. Embryos obtained through bleaching³⁰ were plated on RNAi plates²³ and

grown until L4 larval stage. L4 animals were shifted to new RNAi plates and scored as day 1 adults for vulval bursting using a Leica dissecting microscope.

col-19::gfp expression and seam cell number assay

mir-48 mir-241(nDf51) col-19::gfp (mals105) animals were transferred to RNAi plates as L3 stage larvae and their progeny were scored for heterochronic phenotypes for hypodermal *col-19::gfp* expression. Worms with seam-only reporter expression were classified as having “delayed hypodermal *col-19::gfp* expression”. Seam cell numbers were scored by counting the number of seam cells expressing *col-19::gfp* between pharynx and anus.

Isy-6(ot150) ASEL cell fate assay

Isy-6(ot150); plim-6::gfp and *plim-6::gfp* worms were transferred onto RNAi plates as embryos. Their progeny were scored as L4s specification of ASEL cell fate based on *plim-6::gfp* reporter expression. Worms lacking the reporter expression in ASEL neurons were scored as cell fate defective.

lin-41::gfp reporter assay

Pdpy-30::GFP(PEST)-H2B::lin-41 30 UTR (xeSi78); Pdpy-30::mCherry::H2B::artificial 30 UTR (xeSi36) and *Pdpy-30::GFP (PEST)-H2B::lin-41 30 UTR (xeSi78); Pdpy-30::mCherry::H2B::artificial 30 UTR (xeSi36), let-7(n2853)* embryos obtained by bleaching were plated on RNAi plates and grown at 15°C. Reporter expression was measured in L4 stage animals by imaging the vulva at 63X magnification. To quantify expression levels in six vulval cells, ROIs were manually drawn and signal intensities within the ROI were measured using the Leica image analysis software. For each vulval cell, GFP signal intensity was divided by mCherry signal intensity and relative signal intensities were averaged across the six cells imaged in an individual animal. Representative images were equally adjusted after quantification to make the fluorescence more observable.

hbl-1::gfp reporter assay

hbl-1p::gfp::NLS::hbl-1 3' UTR (ctIS39) and *mir-48 mir-241(nDf51); hbl-1p::gfp::NLS::hbl-1 3' UTR (ctIS39)* embryos were obtained by bleaching. Embryos were transferred to RNAi plates and animals were scored for *hbl-1p::gfp* expression in hypodermal cells at early to mid L3 stage. Animals were staged by time of development, and gonad size and shape.

Uterine cog-1 reporter expression

cog-1::gfp and *pcog-1::Isy-6; cog-1::gfp* animals were transferred onto RNAi plates as embryos and their F1 progeny were scored at L4 stage for *cog-1::gfp* expression in the uterine cells. Worms expressing *cog-1::gfp* in both uterine cells and vulval cells were scored as wild type. Worms that lacked *cog-1::gfp* reporter expression in either of the two uterine cells were scored as abnormal.

Fluorescence microscopy, image capture and illustrations.

Fluorescence equipped Zeiss Axioplan 2 or Leica DM6 upright microscopes were used for scoring phenotypes. Images were captured using the Leica DM6B camera and processed using the associated Leica software. Illustrations in Figure 1A and Figure 6 were drawn using Biorender (biorender.com).

Statistical analysis

All statistics were done using GraphPad Prism (9.2.0 (332)) software. Statistical significance was determined using a one-way ANOVA test with predetermined comparisons. Bonferroni correction was applied as a post hoc analysis. T-test was used to determine statistical significance of *gfp::lin-41*, *gfp::hbl-1* and *cog-1::gfp* reporter assays.

Results

miRNA pulldowns (PDs) identify overlapping sets of putative physical interactors of miRNA-centered complexes.

To identify factors that may regulate miRNA activity, we sought to determine the molecular composition of protein complexes associated with let-7, miR-58, and miR-2 miRNAs. *let-7* is highly conserved across all bilateral animals³¹ and is required for the larval to adult transition in *C. elegans*³². miR-58 is a highly abundant miRNA that regulates lifespan and dauer formation³³, primarily by coordinating with the TGF- β pathway^{34,35}. miR-2, a neuronal miRNA conserved among invertebrates³⁶, is necessary for proper neuromuscular junction function in *C. elegans*³⁷. We used biotinylated, 2'O-methylated oligonucleotides with perfect sequence complementarity to mature miRNA sequence to pulldown miRNAs of interest and characterized the precipitates using a shotgun proteomics approach (Fig. 1a) to identify proteins associated with miRNAs of interest compared to scrambled control (Fig. 1b-d, Supplementary Table S1). To identify high confidence interactors, we retained only the proteins that were ≥ 4 fold enriched in miRNA pulldowns over the scrambled control, had a minimum NSAF value of 4, and were identified in all replicates. Overall, a total of 211 proteins passed the criteria we set (Fig. 1e), with 136 factors co-precipitating with let-7, 54 factors co-precipitating with miR-58, and 25 factors co-precipitating with miR-2 (Fig. 1e, Supplementary Table S1). Among the proteins enriched in miRNA co-precipitates were known miRISC components including ALG-1 and ALG-2, the two major miRNA-associated Argonautes in *C. elegans*³⁸ and AIN-1 and AIN-2, GW182 homologs and miRISC effectors^{39,40} (Fig. 1b-f). In addition, DCR-1 nuclease, responsible for pre-miRNA processing, was detected in all pulldown experiments performed, but did not meet our stringent interaction criteria in the let-7 pulldown (Fig. 1e, f, Supplementary Table S1).

To determine the overlap between complexes precipitated by miRNA pulldowns and those previously found to associate with ALG-1¹⁹, we compared miRNA and ALG-1 co-precipitated factors (Fig. 1e-f, Supplementary Table S1). Eleven (8%) let-7 interactors, ten (18.5%) miR-58 interactors, and five (24%) miR-2 interactors were found to overlap with the ALG-1 co-immunoprecipitated dataset (Fig. 1e, Table 1). Overall, 41 proteins were present in at least two interaction datasets (Fig. 1e, f, Table 1), potentially representing general miRNA-associated co-factors. In addition, four proteins, HRPK-1, SMG-2, IMPH-1, and

SUP-26, were present in 2 out of 3 pulldowns and the ALG-1 IP (Fig. 1e, f, Table 1). Their homologs were also found to co-immunoprecipitate with human and/or mouse Argonautes^{17,18,19}, suggesting that they may have a conserved function in miRNA-mediated gene regulation. In fact, we previously confirmed a physical HRPK-1 interaction with ALG-1 and reported *hrpk-1* to genetically interact with multiple miRNAs⁴¹. In addition, five proteins were commonly captured in all the miRNA pulldowns (Fig. 1e, f, Table 1). Interestingly, one such protein was LET-363, an mTOR homolog⁴² (Fig. 1e, f, Table 1). Similarly, we observed overlaps between our miRNA co-precipitates and previously reported miRNA physical interactors^{43,44} (Supplementary Table S2). Overlaps among our miRNA interaction datasets and AIN-1 and AIN-2 co-precipitates^{40,44} were also observed, further emphasizing that our approach captured potential miRISC interactors (Supplementary Table S2). Finally, candidates identified in genetic screens for miRNA and siRNA pathway genes also intersected with many of our miRNA co-precipitates^{29,45,46} (Supplementary Table S2). The observed overlaps among various groups of physical and genetic interactors support the idea that we are detecting real physical interactors of miRNA-centered complexes.

Table 1

Proteins that co-precipitated with two or more miRNAs or with ALG-1. The table shows the proteins that co-precipitated with either two or more miRNAs in our study and/or identified in previously reported ALG-1 IP¹⁹.

Sequence name	Protein name	Average NSAF ^{pull-down/control} (spectral count)				Description
		let-7	miR-58	miR-2	ALG-1 ¹⁹	
F48F7.1	ALG-1	6 (88)	19 (328)	8 (73)	573 (229)	Argonaute, miRISC component
C06G1.4	AIN-1	5 (25)	16 (83)	11 (28)	125 (122)	miRISC component (GW182 homolog)
C04E6.11	C04E6.11	6 (86)	5 (35)	5 (7)	NA	Unknown
T07D3.7	ALG-2	115 (70)	293 (292)	105 (47)	NA	Argonaute
B0261.2	LET-363	5 (8)	9 (8)	47 (24)	NA	<i>C. elegans</i> Mtor
T23F6.4	RBD-1	20 (124)	5 (55)	27 (28)	NA	rRNA processing
M88.5	IMPH-1	9 (310)	10 (237)	NA	23 (39)	KH domain, RNA binding protein
F26B1.2	HRPK-1	146 (19)	44 (4)	NA	8 (14)	KH domain, RNA binding protein
R10E4.2	SUP-26	139 (20.5)	43 (5)	NA	113 (29)	Translational regulation
Y48G8AL.6	SMG-2	6 (43)	45 (17)	NA	9 (4)	NMD protein
B0041.2	AIN-2	22 (6)	87 (83)	NA	NA	miRISC component
Y49E10.15	SNR-6	7 (41)	5 (19)	NA	NA	Small nuclear ribonucleoprotein
Y71F9B.4	SNR-7	8 (42)	260 (14)	NA	NA	Small nuclear ribonucleoprotein
W08E3.1	SNR-2	9 (48)	5 (19)	NA	NA	Small nuclear ribonucleoprotein
Y116A8C.42	SNR-1	10 (55)	4 (10)	NA	NA	Small nuclear ribonucleoprotein

Average NSAF ^{pull-down/control} (spectral count)						
C52E4.3	SNR-4	381 (31)	4 (13)	NA	NA	Small nuclear ribonucleoprotein
F43H9.3	F43H9.3	4 (23)	52 (8)	NA	NA	Predicted to enable nucleotidyltransferase activity
ZC373.2	ZC373.2	56 (5)	43 (5)	NA	NA	Unknown
W07E6.1	NSUN-1	12 (5)	67 (12)	NA	NA	Nop2 (NOP2)/SUN domain family member
Y38C9A.2	CGP-1	61 (22)	52 (8)	NA	NA	Predicted to enable GTPase activity
K10D2.3	CID-1	16 (53)	14 (6)	NA	NA	RNA 3' uridylation
W05F2.6	W05F2.6	139 (21)	114 (14)	NA	NA	Unknown
T01H10.8	LYST-1	41 (38)	15 (12)	NA	NA	Lysosomal trafficking regulator protein
K04G7.10	RNP-7	36 (14)	66 (5)	NA	NA	RNA binding protein
Y37H2A.1	Y37H2A.1	147 (25)	65 (8)	NA	NA	Predicted to enable hydrolase activity
F56B3.5	ECH-5	94 (20)	64 (12)	NA	NA	Enoyl-CoA Hydratase
F57H12.6	F57H12.6	166 (12)	4 (9)	NA	NA	Unknown
F42A6.7	HRPA-1	30 (5)	14 (4)	NA	NA	RNA binding protein
F25B5.7	NONO-1	84 (14)	100 (13)	NA	NA	Conserved nuclear protein
H20J04.8	MOG-2	161 (34)	NA	114 (6)	NA	Enables U2 snRNA binding activity
W02F12.5	DLST-1	88 (75)	NA	NA	18 (12)	DihydroLipoamide S-SuccinylTransferase
F33D11.10	F33D11.10	16 (7)	NA	NA	29 (4)	RNA helicase activity
ZC434.5	EARS-1	14 (9)	NA	NA	7 (4)	Glutamate-tRNA ligase activity
H05C05.1	H05C05.1	32 (9)	NA	NA	12 (4)	Predicted to enable RNA strand annealing activity

		Average NSAF ^{pulldown/control} (spectral count)				
Y47D3B.10	DPY-18	NA	15 (9)	NA	23 (5)	Procollagen-proline 4-dioxygenase activity
K02F2.1	DPF-3	NA	58 (14)	NA	42 (15)	Serine-type peptidase
F52B5.3	F52B5.3	6 (40)	NA	NA	6 (4)	Predicted to enable ATP binding activity
F18H3.3	PAB-2	NA	20 (9)	NA	5 (69)	Poly-A-binding protein
K12H4.8	DCR-1	NA	153 (84)	27 (10)	6 (5)	Small RNA processor
F37C12.11	RPS-21	NA	NA	89 (4)	39 (33)	Ribosomal protein
Y71F9AL.9	Y71F9AL.9	NA	NA	45 (6)	37 (7)	Unknown

Due to a high level of sequence similarity amongst the *let-7* miRNA family members, the *let-7* complementary oligonucleotide precipitates other members of the miRNA family, albeit with reduced efficiency⁴⁷. To determine whether distinct populations of proteins might associate with *let-7* miRNA family members, we performed additional *let-7* pulldown experiments in *mir-48 mir-241(nDf51); mir-84(n4037)* mutant animals (Fig. 1g, Supplementary Table S1). 55 (29.4%) proteins were in common among both *let-7* pulldowns, suggesting that these factors may interact with *let-7* itself (Fig. 1h). The 51 (27.3%) proteins that precipitated with the *let-7*-complementary oligonucleotide from the *mir-48 mir-241(nDf51); mir-84 (n4037)* animals may similarly represent *let-7*-interacting factors, having been enriched in the *let-7* pulldown in the absence of miR-48, miR-241, and miR-84 (Fig. 1h). In contrast, 81 (43.3%) proteins were present only in wildtype background pulldowns (Fig. 1h), suggesting that these factors may normally associate with miR-48, miR-241 and miR-84 (Fig. 1h). While we cannot rule out an association with the remaining *let-7* family miRNAs, miR-793-795, the low relative abundance of these miRNAs¹⁹ suggests that miR-793-795 interactors are unlikely to represent significant fractions of the observed co-precipitates. Finally, some co-precipitates could represent non-specific interactions.

Ribonucleoprotein complex components are enriched among miRNA interactors.

To understand what biological processes and functions are represented in the miRNA-precipitated complexes, we performed Gene Ontology (GO) analysis on putative miRNA interactors (Fig. 2a-d, Supplementary Table S3). Factors implicated in embryonic and larval developmental processes were commonly enriched in all interaction datasets (Fig. 2a-c; ALG-1 interactome analysis is shown in Fig. 2d for comparison¹⁹, Supplementary Table S3). Selective enrichment for splicing associated factors was observed in *let-7* and miR-58 PD datasets (Fig. 2a, c, Supplementary Table S3). Components of

intracellular ribonucleoprotein complexes were consistently captured in all datasets (Fig. 2a-d and ¹⁹). Enrichment for ribosomal components was observed only in *let-7* and ALG-1 datasets (Fig. 2a, b, d, and ¹⁹). Unsurprisingly, the RNA/nucleic acid binding term was commonly overrepresented in all the datasets in the molecular function category (Fig. 2a-d and ¹⁹). miR-2 interactors did not show an enrichment for any of the GO terms (Supplementary Table S3), potentially due to the low number of interactors captured in our pulldown and were therefore excluded from further analysis.

As RNA binding proteins (RBPs) were enriched in miRNA co-precipitates (Fig. 2a-d) and RBPs carry distinct domains critical for their RNA binding activity, we examined what RNA binding domains (RBDs) were present in proteins identified in our pulldowns and ALG-1 IP (Fig. 2e-h, Supplementary Table S3). At least 8 different RBDs were observed among RBPs in all the datasets. Notably, RBDs such as RNA Recognition Motif, KH, PAZ, and Nucleotide-binding alpha-beta plait domain superfamily were present in RBPs in every dataset (Fig. 2e-h, Supplementary Table S3). Overall, miRNA pulldowns captured factors that may play critical roles during the lifecycle of RNA.

To determine whether factors identified through our proteomics approach form a functional network, we performed network analysis using STRING²⁸ (Fig. 2i-l). STRING predicts candidate protein interactions by utilizing both known and predicted protein-protein interactions sourced from databases, text mining, experimental, and co-expression data²⁸. Top 40 enriched putative interactors in each dataset, minus the ribosomal proteins, were chosen for this analysis. Interestingly, we observed that in all the datasets proteins form functional networks with a significant number of edges (p -value < $1.0e-16$, as determined by STRING) (Fig. 2i-l), further supporting the idea that miRNA pulldown-captured proteins form functional complexes that may coordinate with miRNAs to regulate gene expression.

Functional analysis of putative miRNA interactors

To identify which putative interactors might functionally coordinate with miRNAs to regulate gene expression, we took advantage of sensitized genetic backgrounds with reduced miRNA or miRNA family activity. These functional assays with quantifiable phenotypic outputs allow for assessment of a gene's role in miRNA-mediated gene repression. For functional assessment, we included genes of putative miRNA interactors from this study as well as previously identified ALG-1 interactors¹⁹. We excluded ribosomal proteins and factors lacking RNAi clones.

RNAi knockdown of genes of *let-7* and ALG-1 interactors alters *let-7(n2853)* mutant phenotype.

let-7 is essential for *C. elegans* development and promotes transition from the fourth larval stage (L4) to adulthood³². Loss of *let-7* function results in vulval bursting and failure of seam cells to differentiate during the transition from L4 to adulthood³². *let-7(n2853)* is a temperature-sensitive reduction of function mutation that impairs regulation of *let-7* targets, including *lin-41*⁴⁸. *let-7(n2853)* mutants have a partially penetrant vulval bursting at permissive temperature³² (15°C) (Fig. 3a). RNAi of six genes enhanced vulval bursting of *let-7(n2853)* mutant (Fig. 3b,c, Supplementary Table S4). One such gene encoded a conserved

AGO interactor¹⁹ (Fig. 3b, Supplementary Table S4) and five genes encoded let-7 interactors (Fig. 3c, Supplementary Table S4). RNAi of *cey-1* suppressed the bursting (Fig. 3c, Supplementary Table S4). RNAi of *ifg-1* also suppressed *let-7(n2853)* vulval bursting from 30% in *let-7(n2853)* to 6% (Fig. 3b, Supplementary Table S4). While *ifg-1* RNAi suppression was not statistically significant, it may reveal biological significance. RNAi knockdown of these genes in the wild type background did not result in vulval bursting, suggesting that these genes play an auxiliary role in gene regulation, only revealing the function in the sensitized *let-7(n2853)* background (Fig. 3b, c).

Several genes that affected *let-7(n2853)* vulval bursting at 15°C such as *cey-1*, *ifg-1*, *pab-1* and *rbd-1* in our study, were previously identified in an RNAi based let-7 target screen⁴⁹. While knockdown of *cey-1* suppressed vulval bursting at 15°C in our study (Fig. 3c, Supplementary Table S4), no suppression was observed at 25°C upon *cey-1* knockdown⁴⁹. This suggests that *cey-1* may act in a let-7 dependent manner. On the other hand, *ifg-1* suppressed vulval bursting both at 15°C (Fig. 3b, Supplementary Table S4) and at 25°C⁴⁹, suggesting that *ifg-1* may act downstream of let-7 or that the observed suppression is let-7 independent. Depletion of *pab-1* at 15°C enhanced bursting in our study (Fig. 3b, Supplementary Table S4), consistent with the previously reported role of *pab-1* in miRNA-mediated gene regulation⁵⁰. However, at 25°C, *pab-1* knockdown led to suppression of vulval bursting⁴⁹, suggesting that *pab-1* potentially has miRNA dependent and independent roles in mediating gene regulation during vulval development. Similarly, *rbd-1* knockdown enhanced bursting at 15°C (Fig. 3c, Supplementary Table S4), while it suppressed bursting at 25°C⁴⁹, again suggesting that *rbd-1* could have both let-7 dependent and independent functions. Another interesting finding from this assay was RNAi of mTor homolog gene *let-363*, which enhanced the vulval bursting (Fig. 3c). Previously, RNAi of *let-363* was reported to exacerbate defective phenotype associated with another miRNA mutant, *Isy-6(ot150)*⁵¹, consistent with a functional requirement of *let-363* for miRNA-mediated gene regulation. Overall, these findings support our hypothesis that the identified putative physical interactors may play a role in miRNA-mediated gene repression.

Since dysregulation of *let-7* target gene *lin-41* in vulval-uterine system is sufficient to cause vulval rupturing⁵², we wanted to determine how depletion of putative physical and genetic *let-7* interactors affects *lin-41* expression in the relevant cells. To do this, we used an established *let-7-lin-41* reporter system⁵². We performed RNAi knockdown of genes that enhance (*ech-5*, *rbd-1*, *C04E6.11*, and *let-363*) and suppress (*cey-1*) *let-7(n2853)* vulval bursting in the background of two reporter strains: *Pdpy-30::gfp::lin-41 3'UTR* and *Pdpy-30::gfp::lin-41 3'UTR; let-7(n2853)*⁵². RNAi depletion of these genes did not alter *lin-41* levels in the wildtype background (Fig. 3d, e, Supplementary Table S5), suggesting that these genes do not have a major effect on *lin-41* levels on their own. However, in *let-7(n2853)* background at 15°C, knockdown of *ech-5* or *rbd-1* substantially increased *lin-41* levels, while *cey-1* depletion reduced *lin-41* levels as assayed by reporter expression (Fig. 3d, e, Supplementary Table S5). RNAi of *let-363* led to a mild increase in the *lin-41* levels in *let-7(n2853)* at 15°C, although the increase was not statistically significant (Fig. 3e, Supplementary Table S5). Interestingly, *let-363* depletion was previously reported to increase levels of let-7 target reporters *hbl-1p::hbl-1::hbl-1* in VNC and *col-10::gfp::lin-41 3'UTR* in

hypodermal cells⁵¹. These observations suggest that *ech-5*, *rbd-1*, *cey-1* and perhaps even *let-363* may be contributing to regulation of vulval bursting by modulating *let-7* miRNA activity.

RNAi knockdown of genes of *let-7* and ALG-1 interactors alters *mir-48 mir-241(nDf51)* mutant phenotype.

We next asked whether miRNA and ALG-1 interactors might functionally coordinate with other members of the *let-7* family of miRNAs. *let-7* family members *mir-48*, *mir-84* and *mir-241* specify developmental timing in *C. elegans*, regulating seam cell divisions and contributing to L2-L3 larval developmental transition⁵³ (Fig. 4a). Deletion of all three miRNAs (*mir-48 mir-241(nDf51); mir-84(n4037)*) results in reiteration of L2 stage seam cell divisions resulting in an increased number of seam cells and delayed terminal cell differentiation in young adults⁵³ (Fig. 4a). Partial deletion (*mir-48 mir-241(nDf51)*) mutants display incompletely penetrant heterochronic phenotype which can be monitored using an adult stage marker, *col-19::gfp*, expressed in seam and hypodermal cells (Fig. 4b).

RNAi of 11 genes enhanced the abnormal *col-19::gfp* expression in hypodermal cells (Fig. 4c, d, Supplementary Table S4), while depletion of these genes did not affect *col-19::gfp* expression in the wild type (Fig. 4c, d), suggesting that these potential miRNA interactors are required for regulation of developmental timing programs. We also examined the effects of gene knockdown on seam cell number. RNAi of three genes enhanced seam cell number and surprisingly RNAi of nine genes suppressed the seam cell lineage defect (Fig. 4e, f, Supplementary Table S4). We should note that while most genes were assayed across a minimum of two independent RNAi experiments, four genes (*snr-6*, *snr-4*, *let-363* and *rnp-7*) were tested only once, as knockdown of these genes caused partial lethality, reduced brood size, and slowed growth. Depletion of certain genes had varied effects on hypodermal *col-19::gfp* expression and seam cell lineage, with some genes modifying a *col-19::gfp* expression but not seam cell number and *vice versa* (Fig. 4d, f). This could perhaps be due to distinct roles these genes play during seam cell divisions and hypodermal cell fate specification.

Since *let-7* family miRNAs promote L3 cell fates by repressing *hbl-1*⁵³, we sought to determine whether the genes that affect heterochronic phenotypes in *mir-48 mir-241(nDf51)* background do so by regulating *hbl-1*. We used the *hbl-1::gfp::hbl-1 3'UTR* fusion construct as a reporter to assess the effects of gene knockdown on levels of HBL-1⁵⁴. Strong *hbl-1* expression can be seen during embryogenesis with hypodermal *hbl-1::gfp::hbl-1 3'UTR* expression decreasing beyond detection at the L3 stage⁵⁴ (Fig. 4g). RNAi knockdown of *pdi-2*, *rbd-1*, *Y71F9AL.9*, and *cey-1* resulted in higher percentages of L3 animals expressing the reporter in the wild type background, potentially indicating a miRNA-independent effect (Fig. 4h, Supplementary Table S5). Since depletion of these genes did not affect *col-19::gfp* expression or other heterochronic defects in the wild type (Fig. 4d), perhaps the extent of *hbl-1* derepression was not strong enough to impact developmental timing. This notion is supported by the observation that *hbl-1* reporter expression in *mir-48 mir-241(nDf51)* background is observed at a higher rate (75%) (Fig. 4h). Knockdown of enhancers of *mir-48 mir-241(nDf51)* mutant phenotype derepressed *hbl-1* reporter expression in the *mir-48 mir-241(nDf51)* background (Fig. 4h, Supplementary Table S5). Overall, these

findings suggest that our proteomics approach captured proteins that may co-ordinate with *let-7* family miRNAs to repress their target, *hbl-1*, and ultimately coordinate developmental timing.

Depletion of ALG-1 physical interactors altered *Isy-6*(*ot150*) phenotype.

We hypothesized that some of the ALG-1 and/or miRNA putative physical interactors could be factors that are generally required for miRISC activity. To test this, we RNAi depleted them in *Isy-6*(*ot150*) background. *Isy-6* is essential for cell fate determination of chemosensory ASE neurons⁵⁵. *Isy-6* represses an ASER cell fate promoting transcription factor *cog-1*, leading to an ASEL neuronal specific gene expression pattern. Loss of *Isy-6* leads to dysregulated gene expression of *cog-1* and downstream effectors resulting in defective ASEL cell fate which leads to lack of *plim-6::gfp* reporter. However, the reduction of function mutant *Isy-6*(*ot150*) shows partially penetrant cell fate defective phenotype in approximately 20% of animals⁵⁵(Fig. 5a). Deletion of 8 genes modified *Isy-6*(*ot150*) defective phenotype (Fig. 5b, c, Supplementary Table S4) which included genes of two previously reported conserved AGO interactors *pab-1* and *larp-1*¹⁹ (Fig. 5b). Interestingly, we identified two suppressors of ASEL cell fate defect (Fig. 5b, c, Supplementary Table S4). To determine whether these candidate factors can influence ASEL cell fate independent of *Isy-6* miRNA, we knocked them down in wild-type worms and observed no change in *plim-6::gfp* reporter expression in ASEL cells (Fig. 5b,c).

We next determined whether genes that modified *Isy-6* phenotype upon knockdown were important for *Isy-6* mediated target activity using *cog-1* reporter system⁵⁶ (Fig. 5d). *cog-1* is expressed in vulval and uterine cells where *Isy-6* is absent (Fig. 5d, top left panel). When *Isy-6* is ectopically expressed in these tissues under *cog-1* promoter, there is reduced expression of *cog-1* as a result of *Isy-6* mediated repression⁵⁶ (Fig. 3d, top right panel). We performed RNAi knockdown of top hits from *Isy-6* assay in the *cog-1* reporter strain and observed no difference in *cog-1* expression (Fig. 5e, Supplementary Table S5), suggesting that these factors do not regulate *cog-1* directly in uterine cells. RNAi knockdown of *F33D11.10* and *psf-1* (enhancers of *Isy-6*(*ot150*) phenotype) restored *cog-1* expression in the presence of *Isy-6*, suggesting their requirement for *Isy-6* mediated *cog-1* repression (Fig. 3e). Knockdown of *larp-1* restored *cog-1* expression some of the replicates (Fig. 3e), although the effect was not statistically significant. *Isy-6*(*ot150*) and *cog-1* reporter assays collectively demonstrate that we identified factors that directly or indirectly coordinate with *Isy-6*, affecting its target *cog-1* expression.

Discussion

To better understand miRNA mediated gene regulation, we performed miRNA pulldowns to identify components of miRNA-centered complexes. Our proteomics approach captured 211 miRNA-interacting proteins, some of which were previously reported to precipitate with other miRISC components (Supplementary Table S2). We then sought to determine the functional consequences of the putative interactors. An RNAi screen depleting each of the 39 interactors identified factors that genetically modified reduction of function miRNA backgrounds (Supplementary Table S4). Knockdown of 25 genes significantly modulated miRNA mutant phenotypes in one or more assays, suggesting that our pulldowns

captured proteins that may coordinate with miRNAs to affect gene regulation. Depletion of 23 of these genes, many of which were RNA binding proteins, enhanced the miRNA mutant phenotypes, consistent with the recent finding that mRNA 3'UTR binding RBPs generally promote miRISC targeting⁵⁷. Frequently, depletion of putative interactors did not result in a phenotype in the absence of the sensitized miRNA mutations, suggesting that these factors are not critical for regulation of miRNA target gene expression, but rather have a modulatory role. Genetic interactors of miRNAs identified in this study ranged from RNA binding proteins to metabolic enzymes (Fig. 2a-h, Supplementary Table S3).

How could these putative physical miRNA interactors be coordinating with miRNAs to regulate gene expression? The factors identified in this study could be acting via multiple mechanisms to affect miRNA mutant phenotypes. We used the biological and molecular functions predicted by GO term analysis to consider their possible mode of action. RNA binding proteins were among the classes of genes enriched in our pulldowns (Fig. 2a-h, Supplementary Table S3). Through functional assays, we identified five RBPs as genetic interactors of miRNA mutants (Supplementary Table S4). These RBPs could play a role in miRNA processing (Fig. 6a), while others could potentially facilitate miRISC targeting or activity (Fig. 6b). Consistent with this idea is the overlap among proteins identified in our miRNA pulldowns and those captured in DCR-1 IP and other miRISC component IPs (Supplementary Table S2). In addition, some RBPs could regulate localization and stability of miRNAs and/or miRNA targets, thereby affecting gene regulation.

Translation regulators were also captured in miRNA pulldowns and ALG-1 IP (Supplementary Table S1, S3), with two of them modifying miRNA phenotypes. Depletion of *ifg-1*, encoding translation initiation factor 4G (eIF4G)⁵⁸, suppressed vulval bursting of *let-7(n2853)* and ASEL cell fate defect of *Isy-6(ot150)* (Fig. 3b and 5b). Given the potential physical association of IFG-1 with ALG-1¹⁹, it is possible that IFG-1 and miRNAs share common targets; with loss of IFG-1 reducing translation through loss of initiation, thereby suppressing target mRNA overexpression in miRNA reduction of function mutants. RNA helicase F33D11.10 co-precipitated in *let-7* pulldown (Supplementary Table S1) and was previously identified as an interactor of ALG-1¹⁹. Loss of *F33D11.10* activity enhanced the ASEL cell fate defect of *Isy-6(ot150)* mutant (Fig. 5b). RNA helicases have been previously implicated in miRNA processing as well as miRISC activity⁵⁹ and F33D11.10 may be similarly involved in either facilitating miRNA processing, miRISC activity, or both.

A surprising category of interactors identified in our study was the intermediary metabolic enzymes. RNAi depletion of metabolic enzymes DLST-1, OGDH-1 and ECH-5 modified miRNA mutant phenotypes in our study (Fig. 3c, 4d, 4f, Supplementary Table S4). Several reports have suggested that some metabolic enzymes possess RNA binding functions, previously unidentified due to a lack of conventional RNA binding domains^{60,61,62}. It is possible that the metabolic enzymes identified in our study possess similar dual roles. For instance, *ech-5* encodes a homolog of human AU RNA binding methylglutaconyl-CoA hydratase (AUH)⁶³. In humans, AUH plays a dual role as a hydratase and as an RBP, binding AU-rich elements in the 3' UTR of mRNAs⁶⁴. Other ARE-binding proteins have been previously shown to aid in

rapid degradation through deadenylation⁶⁵. In our study, ECH-5 co-precipitated with let-7 miRNAs and *ech-5* depletion enhanced *let-7(n2853)* vulval bursting phenotype (Fig. 3c, Supplementary Table S1). Thus, we might speculate ECH-5 could bridge miRISC complex interaction with deadenylation machinery, with loss of *ech-5* exacerbating the target mRNA stabilization in miRNA mutant backgrounds (Fig. 6c). How other metabolic genes such as *dlst-1* and *ogdh-1*, key players of TCA cycle^{66,67}, influence gene regulation remains unclear. Thorough investigations into molecular mechanisms by which these factors coordinate with miRNAs in gene regulation will be needed.

Do miRNAs within the same family associate with same set of protein interactors? *let-7* family miRNAs are well-studied in *C. elegans*. The four most abundant members of the *let-7* family, *let-7*, *mir-48*, *mir-84* and *mir-241*, are crucial components of the heterochronic pathway, regulating cell fates during larval development^{32,53}. The miRNAs are thought to function semi-redundantly, with distinct targeting capabilities⁶⁸. Part of their ability to target unique targets could come from discrete protein interactors adding a layer of specificity between a *let-7* family miRNA and its target. Yet not much is known about the protein interacting partners of individual members of this family. By performing pulldowns with a *let-7* specific oligo from wild type and *mir-48 mir-241(nDf51); mir-84(n4037)* background, we began the task of unraveling which interactors may be specific to *let-7* itself or other family members (Fig. 1h, Supplementary Table S1). For example, ECH-5 and C04E6.11 were identified in *let-7* pulldowns from both genetic backgrounds, suggesting that they most likely interact with *let-7* (Supplementary Table S1). RNAi depletion of *ech-5* or *C04E6.11* enhanced the *let-7(n2853)* phenotype but showed no effect on the *mir-48 mir-241(nDf51)* associated phenotypes (Fig. 3c). Thus, it is possible that ECH-5 and C04E6.11 specifically interact with, and provide functional support for, *let-7* itself. CEY-1 was captured in *let-7* pulldowns from both wild type and *mir-48 mir-241; mir-84* mutant background, but depletion of *cey-1* enhanced *let-7(n2853)* vulval bursting and suppressed *mir-48 mir-241(nDf51)* mutant phenotypes (Fig. 3c, 4d). This suggests that *cey-1* may functionally interact with multiple members of the *let-7* family, potentially through distinct mechanisms. Previously, miR-241 complementary oligo pulldown captured CEY-1⁴⁴, although it remains difficult to assess specificity, as miR-241 oligo may capture other members of the family, similar to *let-7*⁴⁷. Overall, we cannot rule out the possibility that some of the factors precipitating in miRNA pulldowns are non-specifically interacting with the precipitating oligo, rather than with the miRNA-centered complexes. However, the high rate of functional relevance of these factors for miRNA-mediated gene regulation suggests that this approach captures auxiliary factors that may coordinate with miRNAs mediated gene regulation.

Declarations

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Author contributions statement

S.H: Study concept and design, performing the experiments, analysis and interpretation of data, prepared figures, manuscript writing. G.S.P: Data analysis. I.V-L: Data analysis and editing manuscript. J.A.W and A.A.V: Proteomics experiments and data analysis. A.Y.Z: Study concept and design, performing the experiments, editing manuscript, technical and material support, study supervision.

Additional information

The authors declare no conflict of interest.

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Figures

Figure 1

Figure 1

miRNA pulldowns (PDs) identify putative physical interactors of miRNA complexes. (a) Protein complexes associated with miRNAs were isolated using 2'-OMe modified oligonucleotides complementary to the miRNA of interest and subjected to MudPIT mass spectrometry analysis. (b-d) Average Normalized Spectral Abundance Factor (NSAF) values of factors identified in pulldown experiments (Y-axis) are plotted against that of corresponding scrambled oligo controls (X-axis) for (b) let-7 PD, (c) miR-58 PD and (d) miR-2 PDs. Highlighted in black are key miRISC components and components of miRNA biogenesis machinery. (e) Venn diagram showing the number of factors that passed a set of criteria to qualify as a putative interactor (for description of criteria, see Methods and Materials). Percentages shown here are percentage of total number of proteins (308) that passed the

criteria in interaction datasets including ALG-1¹⁹. **(f)** Factors common to multiple pulldown experiments include proteins with roles in miRNA biogenesis and activity. **(g)** NSAF values of proteins identified in pulldowns with let-7 complementary oligonucleotide from *mir-48 mir-241(nDf51); mir-84(n4037)* mutant animals, plotted against NSAF of proteins identified in scrambled oligo control. **(h)** let-7 complementary oligonucleotide precipitates a partially overlapping set of factors from *let-7*-family miRNA deletion backgrounds. [¶]Proteins commonly co-precipitated with ALG-1, let-7, miR-58, and miR-2, [#]ALG-1 IP, let-7 PD, and miR-58 PD, ^{\$}let-7 PD, miR-58 PD, miR-2 PD, and [&]ALG-1 IP, miR-58 PD, and miR-2 PD.

Figure 2

Figure 2

miRNA pulldowns identify components of translation machinery and mRNA processing factors, which may form a functional network. **(a-d)** Terms identified through GO analysis for biological processes (top four), cellular components (top four) and molecular function categories (top three) among let-7 PDs in **(a)** wild type, **(b)** *mir-48 mir-241(nDf51); mir-84(n4037)* backgrounds, **(c)** miR-58 PD and **(d)** ALG-1 IP¹⁹. Total number of proteins classified under each term shown adjacent to respective bars. **(e-h)** RNA binding domains identified in factors captured in let-7 PDs from **(e)** wild type, and **(f)** *mir-48 mir-241(nDf51); mir-84(n4037)* backgrounds, **(g)** miR-58 PD and **(h)** ALG-1 IP. **(i-l)** The reproducibly enriched proteins form functional network. Network analysis was performed using STRING²⁸ on top 40 enriched interactors in **(i)** let-7 PD (wild type), **(j)** let-7 PD (*mir-48 mir-241(nDf51); mir-84(n4037)*) **(k)** miR-58 PD and **(l)** ALG-1 IP. Ribosomal proteins were excluded from this analysis. Thickness of edges represents degree of confidence of functional linkages. The number of edges is significantly higher than expected, with a p -value $< 1.0e-16$.

Figure 3

Figure 3

RNAi knockdown of genes encoding ALG-1 and *let-7*-associated factors genetically modifies *let-7(n2853)* vulval bursting phenotype. (a) *let-7(n2853)* mutants show partially penetrant vulval bursting phenotype at 15°C. RNAi of (b) genes of previously reported conserved interactors of AGO¹⁹ and (c) *let-7*-precipitated factors in wild type and *let-7(n2853)* mutants. Each dot represents an independent RNAi experiment. Statistical significance was determined by one-way ANOVA with post hoc Bonferroni correction. (d) Effects of vector and *rbd-1* RNAi on *gfp::lin-41* 3'UTR reporter expression in the vulval cells of wild type and *let-7(n2853)* L4 larvae. Compromised miRNA activity in *let-7(n2853)* background leads to de-repression of *gfp::lin-41* 3' UTR reporter expression levels (quantified in e). (e) RNAi of genes of modifiers of *let-7(n2853)* vulval bursting phenotype affects *gfp::lin-41* 3'UTR reporter expression levels in *let-7(n2853)* background. Each dot represents the relative intensity in an individual worm. Vulval precursor cells used for fluorescence quantification are highlighted with dashed circles. T-test was used to determine statistical significance. Vector = empty vector RNAi control.

Figure 4

Figure 4

RNAi knockdown of genes encoding for putative ALG-1 and miRNA interactors modulates *mir-48 mir-241(nDf51)* seam cell lineage and hypodermal cell fate defects. (a) A schematic representation of seam cell lineages of wild type and *mir-48 mir-241(nDf51)* mutant animals throughout *C. elegans* larval development. (b) Wild type young adult animals express adult cell fate marker, *col-19::gfp*, in seam and hypodermal cells, while *mir-48 mir-241(nDf51)* mutants show lack the *col-19::gfp* expression in the hypodermis as young adults some of the time (quantified in c). (c, d) Effects of RNAi knockdown of genes of (c) conserved AGO¹⁹ and (d) other miRISC interactors on hypodermal *col-19::gfp* expression in wild type and *mir-48 mir-241(nDf51)* mutants. Each dot represents an independent RNAi experiment. (e, f) Effects of RNAi knockdown of genes of (e) conserved AGO¹⁹ and (f) other miRISC interactors on the seam cell number in *mir-48 mir-241(nDf51)* mutant young adults. Each dot represents seam cell number in an individual worm. Statistical significance was determined by One-way ANOVA with post hoc Bonferroni correction. (g) Expression of *hbl-1::gfp::hbl-1 3' UTR* reporter in control and *pdi-2* RNAi in wild type and *mir-48 mir-241(nDf51)* L3 larvae. Arrows indicate hypodermal cells and arrowheads indicate seam cells. (h) RNAi of most genes that genetically modified heterochronic defects in *mir-48 mir-241(nDf51)* mutants significantly affected *hbl-1::gfp::hbl-1 3' UTR* reporter expression in both wildtype and *mir-48 mir-241(nDf51)* backgrounds. Each dot represents an independent RNAi experiment. T-test was used to determine statistical significance. Vector = empty vector RNAi control.

Figure 5

Figure 5

ALG-1 and miRNA interactors genetically interact with *Isy-6(ot150)*. (a) *Isy-6* determines the asymmetric expression of chemoreceptors in ASER and ASEL neurons, with *plim-6::gfp* expression marking ASEL neuronal cell fate. ASEL cell defective phenotype in the *Isy-6(ot150)* animals can be observed by lack of *plim-6::gfp* expression some of the time (quantified in b). RNAi knockdown of (b) conserved AGO¹⁹ and (c) miRNA/ALG-1 interactors in *plim-6::gfp* or *Isy-6(ot150); plim-6::gfp* animals. Each dot represents an independent RNAi experiment. Statistical significance was determined by One-way ANOVA with post hoc Bonferroni correction. (d) *cog-1* expression in uterine cells is repressed by *pcog-1::Isy-6*. RNAi of F33D11.10 depresses *cog-1::gfp* in that background (quantified in e). (e) RNAi of genes that modify *Isy-6(ot150)* phenotype alleviates *Isy-6*-mediated repression of *cog-1::gfp* in uterine cells. Each dot represents an independent RNAi experiment. T-test was used to determine statistical significance. Vector = empty vector RNAi control.

Figure 6

Figure 6

Models for possible mechanisms through which miRNA and ALG-1 interactors affect gene regulation. (a-b) RNA binding proteins found interacting with ALG-1 as well as miRNAs could be involved in **(a)** miRNA duplex processing and/or **(b)** facilitating miRISC activity on the targets by associating with downstream effectors. **(c)** Metabolic enzymes with putative RNA binding ability, such as ECH-5, could bind target mRNAs and promote miRISC activity by recruiting deadenylases and decapping proteins.

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