Regulation of the microRNA-Induced Silencing Complex in *C. elegans*.

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Human Genetics)
in the University of Michigan
2015

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DEDICATION

To my parents, Tom and Suzanne, who let me find my own way and supported me on the paths I chose; to my Grandma Pearl who said “Sure!” to life, and who taught me the value of hard work, self-reliance, and education; and to my “science” parents, Drs. Greg Wadsworth and Amy McMillan, who fostered my first scientific steps and inspired me to become a geneticist.

Finally, I hail the tiny nematode, *C. elegans*, a model organism that has charmed me with its simplicity and fortitude, for its tremendous sacrifice in my hands in the pursuit of advancing scientific understanding!

ACKNOWLEDGMENTS

I am indebted to my colleagues in the Kim lab for their intellectual and moral support. To those who taught me: Vishal Khivansara, who epitomized economy and efficiency at the bench, and who generously gave his time to share the techniques and tricks of molecular biology, Ting Han who was delightfully concise, and a wealth of scientific knowledge and experience, and Allison Billi who exemplified the hard work, intellectual rigor and curiosity of a successful graduate student (Thank you for the Dpy game!). It was through their instruction and examples that these colleagues faceted my graduate tenure to brilliance. I cannot repay Mallory Freeberg in enough chocolate to compensate her for her expertise in bioinformatics, attention to detail, and tolerance for IT questions. It has also been a pleasure to work with Natasha Weiser, Danny Yang, and Charlotte Choi. I would also like to recognize the efforts of our lab technician Da Fang and her daughter DJ who provided reagents with a dose of good company. Thank you also to the staff and fellow scientists in the Department of Human Genetics and the LSI. To my dissertation committee of Ray Chan, Aaron Goldstrohm, John Moran, and Tom Wilson, I thank you for your valuable insight, advice and consideration of my work. I also thank John Moran for his time spent helping me craft my presentation skills. I'd also like to recognize Xantha Karp as an exemplary collaborator. She proved a powerful ally using a genetics-minded approach to add key insights to our projects. Finally, I express my deepest appreciation to my committee chair and mentor, John Kim, who has been the ideal mentor for me. He demonstrates the deepest commitment and curiosity to the discipline, demands the utmost from his students, and more from himself, and is unfailingly and unbendingly committed to helping us help ourselves to be independent, freethinking scientists. Thanks for being tough!

PREFACE

This dissertation embodies the major projects I contributed to as a graduate student over the past six years in the lab of John Kim. The main focus of my research was the characterization of factors that regulate the microRNA-induced silencing complex (miRISC) using the model organism *C. elegans*. Fortuitously, my work was given breadth thought the exploration of factors with opposing roles and different modes of modulating microRNA (miRNA) function: Casein Kinase 2 facilitates miRNA function through posttranslational modification of the miRISC co-factor CGH-1, and *C.elegans* Y-box protein-1 (CEY-1), a conserved RNA binding protein, specifically antagonizes the *let-7* miRNA pathway.

CHAPTER 1 provides an introduction to the mechanics and biological significance of the miRNA pathway, including a summary of the historical significance and utility of the study of miRNAs in *C. elegans* and the importance of understanding miRNA regulation in the context of human disease. This chapter also highlights the gap in our current understanding of how the miRNA effector complex, miRISC, is regulated.

CHAPTER 2 highlights research done in collaboration with Ting Han and Vishal Khivansara. It characterizes the novel role of the highly conserved protein kinase, casein kinase II (CK2), in promoting target binding and repression by miRISC. Ting initiated the project and he and Vishal characterized the role of CK2 in the miRNA pathway using both genetic and biochemical approaches. Ting also identified the conserved miRISC co-factor CGH-1 as a potential CK2 substrate. My contribution to this project was the functional characterization of CK2 modification of CGH-1 in the miRNA pathway. There were several other collaborators on this project including: James Moresco, Patricia Tu, and John Yates who contributed mass spectrometry analyses; Mallory Freeberg who performed computational analysis of miRNA

expression levels; and Xantha Karp and Eric Montoye who provided additional phenotypic analyses. The manuscript, which I am co-first author on with Ting and Vishal, is currently in review at *Proceedings of the National Academy of Sciences*.

CHAPTER 3 represents an independent venture to characterize the role of CEY-1 in the miRNA pathway. Based on an observation made shortly after I joined the Kim lab, the development of this project not only marks an endeavor to understand how CEY-1 antagonizes the activity of the microRNA *let-7*, but also the effort to become proficient in a range of genetic, molecular and biochemical techniques required to interrogate the miRNA pathway in *C. elegans*. I formed several collaborations on this project including: Ting Han and Mallory Freeberg who performed and analyzed CEY-1 HITS-CLIP, respectively; Vishal Khivansara who performed RIP experiments and provided invaluable scientific and technical advice; and Xantha Karp and Eric Montoye who designed and performed *let-7* sister and post-dauer phenotypic analyses.

CHAPTER 4 concludes the dissertation with a discussion of future research directions.

Appended is a description of the identification and characterization of the functional HEN1 ortholog of *C. elegans*, HENN-1. Published in *PLoS Genetics* in 2012. I contributed to this project by characterizing the expression and localization of HENN-1 and ERGO-1 in the *C.elegans* germline by immunofluorescence.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

Below is a summary of the standard conventions that apply for genetic nomenclature in *C. elegans* [1,2] that are relevant for this dissertation.

Genes

Currently all *C. elegans* genes are assigned a sequence name corresponding to the genomic fragment from which they were sequenced, for example, F33A8.3 represents the third gene in the genomic fragment it was identified in. Genes can also be given a second name based on mutant phenotype, gene class, homology, etc… that consists of three or four lowercase italicized letters, a hyphen, and an italicized Arabic number, for example, F33A8.3 is also called *cey-1,* indicating it is the 1st identified member of the *cey* gene class.

mRNA and microRNAs

Messenger RNAs for named genes are written in lowercase italicized letters, for example, the gene and mRNA made from the *lin-41* locus are both called *lin-41.*

Mature microRNAs are written miR- , followed by the number of the *mir* gene from which they are transcribed, in line with conventions described in [3]. A handful of historical microRNAs, which are especially relevant to this dissertation, are referred to by their gene name in lowercase italics. Thus all species of the miRNAs *let-7, lin-4,* and *lsy-6* are indicated thus.

Proteins

The protein product of a gene is the gene name written in non-italic capitals, for example, the protein encoded by *lin-41* is LIN-41.

Mutants

Mutations are named using of one or two lowercase italicized letters followed by an italicized Arabic number, where the letter refers to the laboratory in which the mutation was isolated. Mutations are listed in parentheses after the gene name, for example, *cey-1(ok1805)* or *let-7(n2853).* Multiple mutations in a single background are listed by order of the chromosome they reside on, separated by a semicolon, for example, *cey-1(ok1805)*; *let-7(n2853)* indicates the double mutant, since *cey-1* is on chromosome II and *let-7* is on chromosome X.

Phenotypes

A phenotype can be described in words or abbreviated with a 3 or 4 letter non-italicized designation, where the first letter of a phenotypic abbreviation is capitalized, for example, phenotypes described in this dissertation include Rup for vulval rupture, Muv for multiple vulva, and Emb for embryonic lethality.

REFERENCES

1. Horvitz HR, Brenner S, Hodgkin J, Herman RK (1979) A uniform genetic nomenclature for the nematode Caenorhabditis elegans. Molecular & general genetics : MGG 175: 129-133.
2. WormBase (ws248). wormbase.org. accessed 06-04-2015.
3. Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, et al. (2003) A uniform system for microRNA annotation. RNA 9: 277-279.

CHAPTER 1

Introduction to the microRNA field and regulation of the miRNA effector complex, miRISC

ABSTRACT

Over the past two decades microRNAs (miRNAs) have emerged as pervasive, conserved, post-translational regulators of gene expression that function in diverse biological settings. While significant strides have been made in characterizing the biogenesis and gene silencing mechanisms of miRNAs, the regulation of miRNA activity is less well understood. Foremost, a gap in knowledge exists in understanding how the conserved, ribonucleoprotein miRNA effector complex, called the miRNA-induced silencing complex (miRISC), is regulated. The importance of closing this gap is underlined by the essential nature of miRNAs in controlling diverse biological processes, including cell proliferation and differentiation, signaling, metabolism and apoptosis [1,2], and the disease-associated outcome of their dysregulation [3,4].

Historically *C. elegans* has been an important model system for the study of miRNAs: the first miRNAs, *lin-4 and let-7,* were discovered in genetic screens for factors that controlled the timing of cell division and differentiation [5,6,7]. Today the robust and facile genetics of *C. elegans* and the ability to use it in high-throughput methods continue to make the worm an attractive in vivo system for characterizing factors that regulate miRNA silencing.

The work presented in this dissertation describes the identification and characterization of two factors that regulate miRNA silencing in *C. elegans*: the conserved serine/threonine protein kinase CK2 and the RNA binding Y-box/cold-shock domain protein CEY-1.

Conservation and biological importance of microRNAs

MiRNAs are a highly conserved group of post-translational regulators that are involved in crucial processes during animal development, including the differentiation of embryonic stem cells, body patterning, and organ formation [1,3]. Regulation by miRNAs has also been associated with areas important to human health, including longevity [8], learning and memory [9,10,11], and disease [1,3]. Notably, recent studies in mouse models of human disease indicate that aberrant miRNA function [12] or mutations in their processing factors [13,14] contribute to the pathogenesis of common diseases, including cancer, cardiovascular disease [15], and type 2 diabetes [16]. Therefore, a detailed understanding of how miRNAs regulate gene expression holds the promise of informing our ability to prevent, diagnose, and treat common human diseases.

To date, over 35,000 miRNAs have been discovered in 223 species, including more than 2,500 miRNAs in human [17]. In addition to being abundant, genome-wide studies estimate that miRNAs may target up to half of the human transcriptome [18]. Remarkably, despite over 500 million years of evolutionary divergence, about one-third of the approximately 250 identified miRNAs in *C. elegans* share sequence homology with vertebrate miRNA families [19]. Homologous miRNAs, within and between species,

have been grouped into families based on conservation of the “seed” sequence (nucleotides 2–8 at their 5′ end), which provides specificity to miRNA regulation [19,20]. Members of the same miRNA family often have similar spatial and temporal expression patterns, suggesting that their sequence conservation is tied to a common regulatory function [21,22,23,24,25]. A striking example of miRNA conservation is the *let-7* family, which is highly conserved from *C. elegans* to humans [22]. There are thirteen *let-7* family members in human and nine in *C. elegans* [26,27]*;* human *let-7a* has perfect complementarity with *C. elegans let-7* (Fig 1-1A). The *let-7* family has conserved functions in regulating cellular differentiation and development. In *C. elegans, let-7* and its family members, *mir-48*, *mir-84*, and *mir-241*, have well characterized roles in promoting *C. elegans* larval development [28]. *D. melanogaster* development is also regulated by its single *let-7* [29,30,31] and in other organisms *let-7* expression increases during development [21,22,23].

MicroRNAs in human disease

In view of the essential nature of miRNAs in regulating critical biological processes, it is not unexpected that aberrant miRNA expression could affect those processes with pathological results. While a wide range of studies have correlated miRNA dysregulation with disease states, causality of miRNA dysfunction is not always clearly established. Nevertheless, a survey of several common diseases that have evidence of miRNA dysregulation include cancer, neurodegenerative, immunological, and cardiovascular disorders, and diseases associated with viral infections (Table 1-1). The need for improved therapeutic intervention in all of these examples would greatly benefit

from a better understanding of the mechanisms and regulation of miRNA silencing activity.

Compelling work recently demonstrated that distinct miRNA expression profiles were associated with specific cancer types, and reflected the developmental lineage and differentiation state of tumors [32]. In agreement, previous studies reported that human miRNAs were frequently located in fragile sites and deleted or misexpressed [33,34], and scores of subsequent profiling studies suggest that tumors universally exhibit miRNA dysregulation [35]. In addition to specific miRNAs, mutations in miRNA machinery are also implicated in cancer. Haploinsuficiency caused by mutations in *DICER*, an enzyme required for miRNA biogenesis, is correlated with a variety of nonepithelial ovarian tumors, presumably due to a loss of miRNA-mediated gene regulation [36].

Consistent with its conserved role in regulating cell proliferation and differentiation during development, the dysregulation of *let-7* is a feature of many types of cancers [37]. MiRNAs, like *let-7*, that function to balance of cell proliferation and differentiation exercise considerable influence in cancer networks, acting as tumor suppressors (*e.g*. *let-7,* miR-15-16, miR-26a, miR-146a) or oncogenes/oncomiRs (*e.g*. mir-17-92, -21, -29, -155) [38,39]. Extensive research in lung and other cancers show that *let-7* is commonly down-regulated, resulting in increased expression of several key target mRNAs, including the oncogenes *RAS* and *HMGA2* in lung cancer cells and *MYC* in Burkitt lymphoma cells [40,41,42,43,44,45,46].

Direct evidence for miRNA function in tumor formation comes from recent in vivo mouse studies that demonstrate that artificial induction of miR-21 and miR-155 is

sufficient to induce lymphomagenesis [12,47], and that expression of the miR-17-92 polycistronic miRNA accelerates Myc-induced B cell lymphomagenesis [48,49]. Conversely, therapeutic delivery of tumor suppressor miRNAs has been found to effectively inhibit tumor formation [50]. For example, systemic delivery of the *let-7* tumor suppressor miRNA inhibits lung tumors in mice [51], and viral delivery of miR-26a inhibits hepatocarcinoma in mouse models [52].

Thus, understanding how miRNA-mediated gene silencing is regulated promises to aid our understanding of the etiology of common human diseases, perhaps revealing new avenues for diagnosis, treatment, and, ideally, prevention.

Historical significance of miRNAs in *C. elegans*

C. elegans lin-4 and *let-7* were the first identified miRNAs [5,6,7]. Although *let-7* was discovered second, it was its deep sequence conservation and common temporal expression patterns among metazoans that first suggested that miRNAs were not a *C. elegans*-specific phenomenon [22]. *lin-4* and *let-7* were initially discovered in forward genetic screens designed to identify genes that control developmental timing and differentiation (*i.e*. the “heterochronic” pathway) [5,6,7]. *C. elegans* have invariant cell lineages that have been completely mapped from embryo to adult [53,54]. Thus, the temporal displacement of specific lineage events can be identified relative to the boundaries established by the transition between each of the four larval stages (L1, L2, L3 and L4), which are punctuated by a molting cycle.

let-7 was identified as a retarded heterochronic factor whose mutant phenotype suggested it promoted the L4-to-adult-transition [7]. *let-7* null mutations are not viable

(hence *"let"-7* for lethal), however studies of a series of weak mutations (*i.e*. hypomorphs) of *let-7* indicate that reduced *let-7* function results in the failure to adopt adult cell fates. Specifically, *let-7* hypomorphs exhibit reiteration of larval molting and hypodermal seam cell division patterns and compromised vulval integrity. The latter defect results in a dramatic ruptured vulva phenotype, where animals herniate through their vulva and ultimately die [7]. Genetic and molecular evidence suggest that the most biologically relevant target of *let-7* in *C. elegans* is *lin-41* [55], which encodes a TRIM/RBCC family protein homologous to human TRIM71 [56], that negatively regulates expression of LIN-29, a transcription factor required for adult cell fate specification [57] (Fig 1-1B). Hence, *let-7* regulation acts as a developmental switch: beginning in late L3, *let-7* levels rise dramatically, triggering the rapid degradation of *lin-41* mRNA at the L4-to-adult transition [57,58] (Fig 1-1C). Other *let-7* targets include the *C. elegans* hunchback ortholog, *hbl-1* [59], and mRNAs encoding several transcription factors, including the nuclear hormone receptor *daf-12*, the forkhead transcription factor *pha-4,* and the zinc finger protein *die-1* [60]. The homolog of the oncogene *RAS*, *let-60*, is also regulated by the *let-7* family [41].

Three other *C. elegans let-7* family miRNAs have been characterized in the regulation of *C. elegans* development: mir-48, mir-84, and mir-241 [28] (Fig 1-1A). These *let-7* "sisters" cooperatively function in a temporally distinct manner from *let-7*, promoting the L2-to-L3 transition, through the negative regulation of *hbl-1* and *daf-12* [7,28,61] (Fig 1-1B).

C. elegans: a powerful model system for studying the miRNA pathway

In addition to the insights gained by characterizing *let-7* in *C. elegans, let-7* has also proven a useful genetic tool for identifying additional genes required for the miRNA pathway. Hypomorphic alleles of *let-7* provide obvious observable phenotypes that can be enhanced or suppressed to identify genes that positively or negatively affect the miRNA pathway, respectively. To date, several screens have identified genes encoding novel factors, including additional *let-7* targets [62], the exonuclease XRN-2, which accelerates the turnover of mature *let-7* molecules [63], and genes involved in circadian rhythms [64], cell cycle regulation [65], and membrane trafficking [66]. Genome-wide RNAi screens [65,67,68,69] have also identified a host of candidates, the majority of which await molecular characterization.

In addition to *let-7*-based assays, several other miRNAs in *C. elegans* have known targets, and mutants with distinct phenotypes that can also be used to investigate miRNA function: miR-1, a conserved muscle-specific miRNA, regulates targets that affect pre- and post- synaptic function at neuromuscular junctions [70]; *lin-4* regulates the timing of fate specification in neuronal and hypodermal lineages in early larval development [5,71]; and *lsy-6* specifies the neuronal fate of one of two bilaterally symmetric chemosensory neurons [72].

Since miRNAs have largely conserved machinery for their biosynthesis, processing, and silencing functions, factors identified in *C. elegans* commonly have functional equivalents in other species. Therefore, despite its simplicity, *C. elegans* is a powerful tool for in vivo analysis of the miRNA pathway.

MicroRNA biogenesis and post-translational gene silencing

Canonical miRNA biogenesis

Most miRNA genes are initially transcribed by RNA polymerase II as long primary transcripts (pri-miRNA) [73,74] (Fig 1-2A). Like mRNAs, pri-miRNAs are capped, polyadenylated, and may be spliced [73,75]. Pri-miRNAs undergo sequential processing by the RNase III enzymes, Drosha and Dicer. First, within the pri-miRNA, a predicted stem-loop hairpin structure is excised by Drosha (Pasha in fly and worm), to yield a ~70 nt precursor miRNA (pre-miRNA) [76,77,78] (Fig 1-2A). Drosha cleavage is directed by DGCR8 (DiGeorge critical region gene 8), which recognizes the point where the single-stranded transcript transitions into the double-stranded hairpin [79]. Alternatively, some pre-miRNAs are produced from very short introns, known as mirtrons, as a result of splicing and debranching, and therefore bypass the Drosha-DGCR8 processing step [80]. Pre-miRNAs are transported into the cytoplasm by Exportin-5, a member of the karyopherin family of proteins, which mediates transport between the nucleus and cytoplasm, by a Ran-GTP-dependent mechanism [81,82,83]. Pre-miRNAs are further cleaved into a ~22 nt miRNA duplex by Dicer [84,85,86], which cooperates with the TRBP (human immunodeficiency virus transactivating response dsRNA binding protein; Loquacious in fly) [87,88,89] (Fig 1-2A). Finally, the miRNA strand with the lower relative base-pairing stability at its 5′ end is preferentially incorporated as the mature miRNA into an Argonaute protein, which forms the core of the miRNA-induced silencing complex (miRISC) [90,91] (Fig 1-2A). The heat shock proteins HSP70 and HSP90 mediate the ATP-dependent loading of miRNA duplexes by Argonautes [117]. MiRNA loading is also facilitated by DICER, TRBP, and PACT (protein activator of the interferon induced protein kinase), which form a miRNA-loading complex [92,93,94].

Components of the miRNA-induced silencing complex

The Argonaute protein that forms the core of miRISC, AGO1-4 in humans, AGO1 in *Drosophila*, and ALG-1/2 in *C. elegans* [84,95,96], are part of a highly conserved family of small RNA binding proteins that are essential components of RNA-silencing pathways, including the miRNA, piRNA, and endogenous and exogenous siRNA pathways [97]. Despite their unusual moniker, Argonaute proteins were named using traditional genetic nomenclature: the *Arabidopsis* AGO1 mutant phenotype resembles the octopus *Argonauta argo* [98]. Incidentally, the octopus was named based on folk tales that female of the species could glide across the ocean's surface using her egg cases as a boat and her webbed legs as sails, recalling the Argo, a giant wooden sailing vessel of Greek mythology, manned by Jason and the Argonauts [99].

The miRNA-bound Argonaute associates with several protein cofactors to form miRISC. The glycine-tryptophan (GW) protein of 182 kD (GW182; AIN-1/2 in worms) is an important scaffolding factor that is responsible for localizing Argonaute to P-bodies, major cytoplasmic centers for mRNA catabolism, which co-localize with the decapping factors DCP-1/2 and the 5'-to-3' nuclease XRN-1 [100,101,102,103,104,105]. In *C. elegans*, ALG-1 and AIN-1/2 are required for miRNA-mediated translational repression and mRNA target degradation [106] (Fig 1-2B). Work in mammalian systems revealed that the GW motifs of GW182 proteins associate with the CCR4/NOT deadenylase complex and the PAN2/3 nucleases, which function in translational repression [107,108]. GW182 homologs also associate with the EDD E3 ubiquitin ligase, which associates with the Transducer of ERBB2 EGF Receptor (TOB1/2) and the Dead Box helicase DDX6 (CGH-1 in worms) [109]; TOB1/2 acts to physically link CCR4 and the PolyA

Binding Protein (PABP) to promote deadenylation [110], and DDX6 promotes miRISC by interaction with the CCR4-NOT and other mRNA degradation machinery [111,112,113]. In *C. elegans*, the DDX6 ortholog, CGH-1, and the TRIM-NHL ubiquitin ligase NHL-2 are also required for effective miRISC target silencing [114]. In mammalian systems the NHL-2 homolog, TRIM32, has been shown to enhance the activity of *let-7* bound miRISC [115]. Lastly, proteomic analyses in *C. elegans* have identified additional factors associated with miRISC, including the fly Vasa Intronic Gene ortholog, VIG-1, and the Tudor domain protein, TSN-1, which are both required for silencing of a *let-7* target 3' UTR reporter [116].

Mechanisms of miRNA-mediated target silencing

Once incorporated into miRISC, the miRNA provides target specificity; miRNAs identify mRNA targets through base pairing interactions with partially complementary sites predominately located in the 3'UTR of the target mRNA, then miRISC directs their silencing [117,118,119]. Thus, loss of miRNA regulation leads to elevated mRNA and protein expression of targets. If target up-regulation results in a physiological or pathological phenotype it can be ameliorated, in an experimental setting, using RNAi-based strategies to reduce target levels.

MiRISC has been reported to affect silencing by both translational repression and/or mRNA decay (Fig 1-2B). However, a global rule governing the temporal order and/or relative contribution of the silencing mechanisms has not been established [120]. In support of translational repression, ribosome profiling in *C. elegans* and in human cells suggests that upon loss of *let-7* its target mRNAs shift from monosomes to polysomes, suggesting that *let-7* blocks translation initiation [106,121]. Interestingly, the

shift is 5' cap-dependent [122,123], consistent with evidence that the human Argonaute, Ago2, can compete with eIF4E, a translation initiation factor, for cap binding [124]. In agreement, luciferase reporter studies indicate that translational repression often occurs in the absence of mRNA degradation, or before the onset of mRNA degradation [125,126,127].

In contrast, global profiling of actively translating ribosomes suggests that decreased levels of target mRNAs may account for much of the silencing attributed to miRISC [128,129]. Further support for miRNA-mediated target degradation stems from several reports that connect deadenylation and subsequent decapping with miRISC silencing [125,130,131,132,133]; through GW182-mediated interactions, miRISC associates with the polyA-binding protein PABPC [100,104,134,135] and the CCR4-NOT deadenylase complex to initiate polyA tail shortening and degradation [130,134] (Fig 1-2B).

Intriguingly, studies in zebrafish suggest that miRNA-mediated deadenylation in pre-gastrulation-stage embryos triggers translational repression without RNA decay, while RNA decay accompanies decreased translation in later stages and in vitro [146-149]. Notably, mRNA poly-A tail length is positively correlated with translation rate in early embryos, which disappears after gastrulation [147]. Therefore, it appears that miRNA-mediated silencing may occur by different mechanisms during development. While it is evident that several mechanisms are employed to achieve miRNA-mediated silencing, it is likely that both mRNA degradation and translational repression contribute to miRNA target silencing. However, what dictates the predominant means of silencing

appears complex and may be dependent on the miRNA and target, experimental system and/or the developmental context.

Regulation of the miRNA-Induced Silencing Complex

Factors that regulate miRNAs have been identified at nearly every step in the pathway [136]. Due to the scope and focus of this dissertation, emphasis will be placed on the describing what is known about the regulation of miRISC, specifically on recent findings that highlight the underexplored areas of post-translational modification to miRISC and the interaction of miRISC with RNA binding proteins.

Regulation of miRISC through post-translational modification

Post-translational protein modifications play important roles in regulating protein stability [137], protein-protein interactions, and cell signaling and regulatory processes [138]. A limited number of reports functionally implicate post-translational modification in the regulation of miRISC [139]. Understandably, particular focus has been devoted to the modification of Argonaute as it is the central component of miRISC. Phosphorylation of human Ago2 at S387 and Y529 has been demonstrated to affect P-body localization [140,141,142], modifications at Y529 also affect miRNA binding to Ago2 [140], and those at S387 affect miRNA-mediated translational repression [142]. Hydroxylation of human Ago2 at P700 has also been shown to be required for its stability, P-body localization, and silencing activity [143,144]. Additionally, ubiquitination of mouse Ago2 by the TRIM-NHL protein Lin41 promotes its proteasome-mediated degradation [145]. Finally, ADP-ribosylation of all 4 human Agos has been reported to negatively regulate miRNA-mediated silencing under stress conditions [146]. Apart from Argonautes, there is scant evidence supporting functional post-translational modifications of miRISC.

Although phosphorylation of the human GW182 protein has been shown to reduce the efficiency of its association to PABP and subsequent silencing by miRISC [147]. Existing studies mainly support the consensus that post-translational modification can modulate protein stability and localization; few identify modifications that regulate miRISC activity. Therefore, the question of how miRISC is regulated by post-translational modification remains a gap in our understanding of miRNA function.

mRNA regulation by miRISC and RNA binding proteins

A host of RNA binding proteins (RBPs) associate with the untranslated regions of mRNAs to regulate translation, stability, and turnover. The 3'UTR has proven a particularly rich region for binding and regulation. AU-rich elements (AREs), located in the 3'UTR, are bound by RBPs that affect transcript stability. For example, the RBPs AUF/hnRNP-D (AU-Rich Element RNA Binding Protein/heterogeneous nuclear ribonucleoprotein D), TTP (tristetraprolin), and KRSP (K homology Splicing Regulatory Protein) destabilize mRNA by recruiting deadenylation and decay factors [148,149,150], while others such as the Hu/ELAV family proteins primarily function to stabilize transcripts by promoting translation and competing with negative regulatory factors [151,152]. The highly conserved PUF (Pumilio-fem-3 mRNA binding Factor) family proteins also elicit translational repression through association with specific motifs in the 3'UTR of target mRNAs [153].

As the primary site of miRNA regulation, the 3'UTR of miRNA-targeted mRNAs may also be occupied by RBPs that may, directly or indirectly, affect miRNA-induced silencing. The interaction of miRNAs and RBPs has been referred to as the "post-transcriptional regulatory code" [154]. However, despite having such a definitive

description, this “code” is poorly understood. Foremost, only a relatively small proportion of proteins with RNA binding domains have been functionally characterized. It is estimated that RNA sequence motifs are known for only ~15% of human and ~3% of *C. elegans* proteins that have identified RNA-binding domains [155]. Thus, further characterization of the RBP-miRNA interactome is required to fill the gap in our understanding of post-translational regulatory dynamics.

Several key studies have highlighted the importance of miRNA-RBP interactions, characterizing RBPs that affect miRISC activity. For example, DND1 (DND miRNA-mediated repression inhibitor 1) prevents target mRNA silencing by several miRNAs, including miR-221 and miR-430, in human and zebrafish primordial germ cells by binding to mRNA sequences that overlap with miRNA binding sites, which prevents miRISC accessibility and silencing [156]. In contrast, PUF family proteins, which are negative post-transcriptional regulatory factors, are implicated in facilitating miRISC. In *C. elegans*, PUF-9 promotes *let-7* repression of a shared target mRNA [157] and interacts with miRISC in an RNA-dependent manner (Yang, D. *et al.*, unpublished data). This relationship may be conserved as targets of human PUFs are enriched for miRNA sites near PUF binding motifs [158].

Interestingly the nature of the relationship of some RBPs with miRISC appears to be dependent on the proximity of their binding sites. For example, HuR/ELAV has been extensively characterized as a miRISC antagonist [159]. However recent studies that compared transcriptome-wide HuR and miRNA interactions on co-targeted mRNAs suggest a distance-dependent regulatory relationship [160,161]: when HuR/ELAV binding sites overlap with miRNA target sites, HuR generally prevents miRISC

accessibility and silencing, whereas more distally located binding sites are primarily regulated by miRISC. Intriguingly, several studies have reported that HuR facilitates miRISC silencing of specific shared targets when their respective binding sites are non-overlapping, including *MYC, p16,* and *RhoB* mRNAs [162,163,164]. Two additional examples of RBPs that differentially modulate miRISC are PTB (polypyrimidine-tract-binding protein) and the helicase MOV10 (Moloney leukemia virus 10). PTB competes with miRISC when their binding sites overlap but appears to facilitate miRISC access by melting secondary structure when their binding is adjacent [165]. MOV10 facilitates miRISC binding by unwinding G-rich secondary structure, but limits miRISC accessibility on mRNAs also bound by the Fragile X Mental Retardation Protein (FMRP) [166].

Identification of novel miRISC regulatory factors

The work presented in this dissertation describes the identification and characterization of two factors that regulate miRNA silencing in *C. elegans* in opposing fashions: Casein Kinase II (CK2) promotes miRNA function while the RNA binding protein *C. elegans* Y-box Protein 1 (CEY-1) antagonizes it.

Casein Kinase II (CK2)

Named for its misidentification as a casein kinase, CK2 is a serine/threonine kinase that is ubiquitously conserved in eukaryotes. Over the past three decades an impressive amount of research has been dedicated to defining the molecular, biochemical and physiological functions of CK2 (reviewed in [167,168,169,170,171,172]). Dubbed the “master regulator” of cell function, biochemical and functional analyses suggest that CK2 is capable of phosphorylating over 300 proteins in human that are implicated in the regulation of many cellular functions, including protein and nucleic acid synthesis, cell

growth, proliferation and death, signal transduction pathways, neuronal function, angiogenesis, and organogenesis [167,173]. Unsurprisingly, given its involvement in such a diverse range of essential processes, genetic analysis has demonstrated that CK2 is essential for cell viability in yeast, embryonic development in mice, and for post-embryonic development in *C.elegans* [174,175,176,177].

In animals, CK2 functions in a hetero-tetrameric complex composed to 2 regulatory β subunits and two catalytic α subunits arranged in an α2β2 configuration [178] that phosphorylates substrates harboring the S/TXXD/E motif. However, unlike many kinases, which require activation (*i.e*. priming phosphorylation) and function in networks or cascades, CK2 appears to require no such activation and thus has been traditionally defined as a constitutively active kinase [167]. Therefore, how (and if) CK2 is regulated is a critical and yet unresolved question, highlighted by a large body of literature reporting CK2 function in disease and cancer biology.

We first identified CK2 in a genome-wide RNAi screen to discover novel factors in small RNA-mediated gene silencing pathways in *C. elegans* [68]. A relationship between CK2 and the miRNA pathway has not been reported. Since the significance of post-translational modifications to proteins in the miRNA pathway is not well understood, we employed a combination of genetic and molecular techniques to investigate CK2 function in the miRNA pathway. Our findings are the subject of **CHAPTER 2: Casein kinase II promotes target silencing by miRISC through direct phosphorylation of the DEAD-box helicase CGH-1**. In short, we find that CK2 genetically and physically interacts with miRISC and promotes miRISC target association. We identify the conserved miRISC component, the DEAD-box helicase CGH-1, as a direct CK2

substrate and demonstrate that CK2 phosphorylation of CGH- 1 is important for its function in the miRNA pathway.

***C. elegans* Y-box Protein 1 (CEY-1)**

CEY-1 is part of a family of 5 RNA binding Y-box proteins in *C.elegans* that also includes CEY-2, CEY-3, CEY-4, and LIN-28. Over two decades ago, the CEYs were identified by domain homology [179]. However, until recently, little was known about their function in *C. elegans*. In contrast, Y-box proteins (YBPs) have been extensively characterized in other model systems. YBPs function in a variety of cellular processes that involve the regulation of mRNA stability, translation, and/or localization. Notably, YBPs are essential for proper gametogenesis, as characterized in a host of model organisms, including YB1 in human [180], YBX2/MSY2 in mouse [181,182], FRGY2 in frog [183], Ypsilon schachtel (Yps) in fly [184], Ybx1 in fish [185], and CEY-2/-3 in worm [186].

The somatic function of Y-box proteins has been most extensively explored in the human Y-box protein, YB-1/YBX1. Since YB-1 overexpression in multiple human cancers is associated with increased proliferation, tumor aggressiveness, and drug resistance (reviewed in [187,188]), the mechanism and regulation of its functions are of therapeutic interest. YB-1 has been implicated in a range of biological processes, including DNA repair, stress response, and, most notably, in the regulation mRNA transcription, splicing, translation and stability [187,189]. In general, YB-1 appears to promote mRNA stability and translation: it binds mRNA and associates with mRNP granules [190,191], sites of mRNA sequestration and storage, and has also been shown to positively regulate translation by transporting mRNA to polysomes [192].

We first became interested in CEY-1 when we observed that loss of *cey-1,* but not other *ceys*, potently suppressed lethal phenotypes associated with loss of the miRNA *let-7*. Recent findings have highlighted the importance of increasing our understanding of the interplay of RNA binding proteins and miRISC in mRNA regulation. Since Y-box proteins have yet to be connected to miRNA regulation, we investigated the role of CEY-1 in the miRNA pathway. Complementary genetic, biochemical, and molecular analysis of CEY-1 suggest that it specifically antagonizes *let-7* miRISC activity, possibly through association with the 3'UTR of *let-7* targets. Our findings are discussed in detail in **CHAPTER 3: CEY-1 attenuates *let-7* microRNA-mediated gene silencing in *C. elegans.***

SUMMARY

MiRNAs are highly conserved negative regulators of gene expression that influence a wide range of cellular and developmental events. Their essential involvement in a wide range of biological processes, including cellular proliferation and differentiation, has also implicated miRNAs in human disease. Although fundamental insights have illuminated the mechanisms of miRNA biogenesis and gene regulation, we have only a rudimentary understanding of how the miRNA effector complex, miRISC, is regulated. In this regard, post-translational modification to miRISC and the cooperative regulation of a common set of mRNAs bound by miRISC and other RNA binding proteins are two underexplored areas of interest. Historically, *C. elegans* has played a significant role in the discovery and characterization of miRNAs and the miRNA pathway. Today *C. elegans* continues to be a powerful model system for identifying factors that regulate the miRNA pathway.

The work presented in this dissertation describes the identification and characterization of two novel factors that regulate miRISC in *C. elegans,* Casein Kinase II and CEY-1.

Figure 1-1. *let-7* family miRNAs in *C. elegans*. (A) Alignment of mature human *let-7a* and the members of the *C. elegans let-7* family. The miRNA seed is indicated in red. *let-7* family members with characterized functions in regulating *C. elegans* development, in green. **(B**) Genetic diagram of the *let-7* regulatory network. miRNAs are in green, their mRNA targets are in black. **(C)** Cartoon depiction of *let-7* regulation of *lin-41* mRNA at the L4 to adult transition.

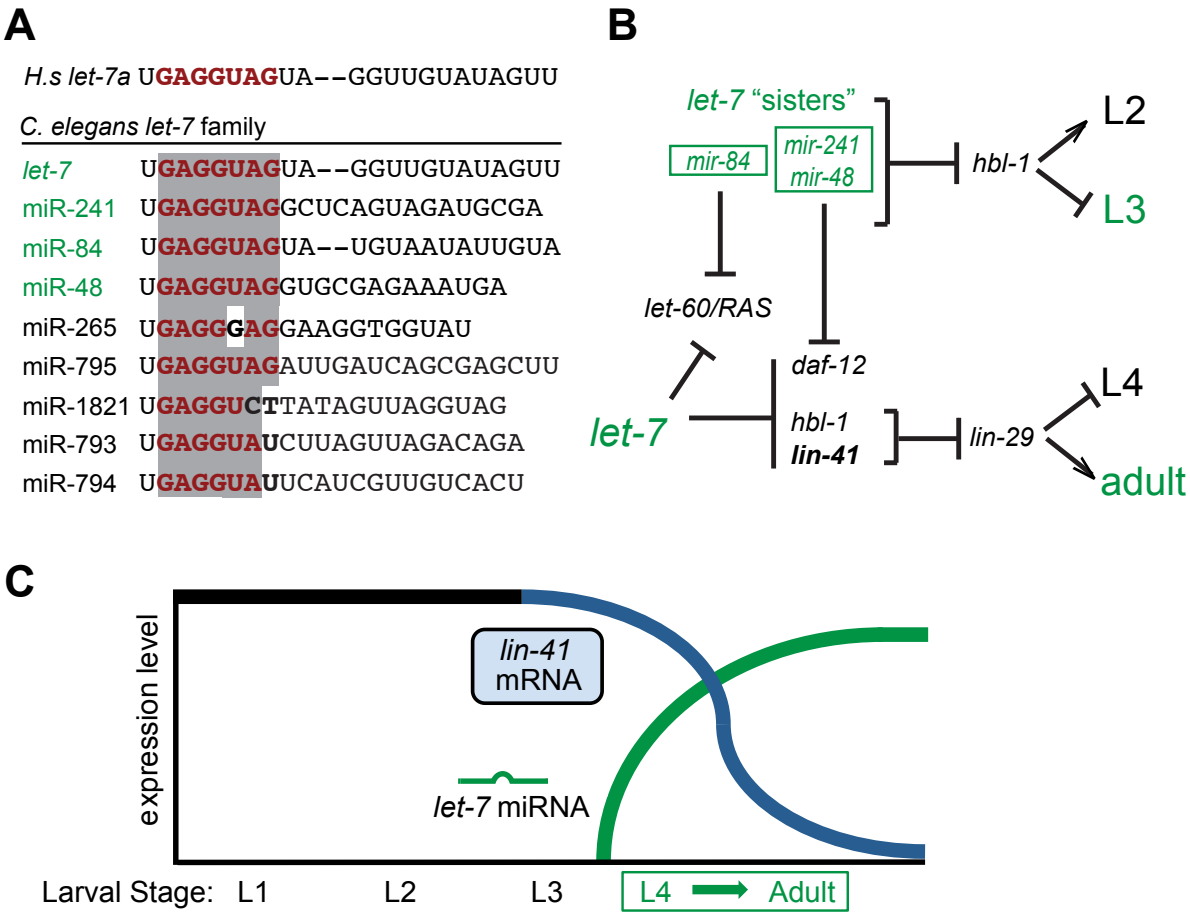

Figure 1-2. The miRNA pathway. (A) Cartoon depicting the major steps in miRNA biogenesis. **(B)** Cartoon depicting miRISC binding and silencing of an mRNA target.

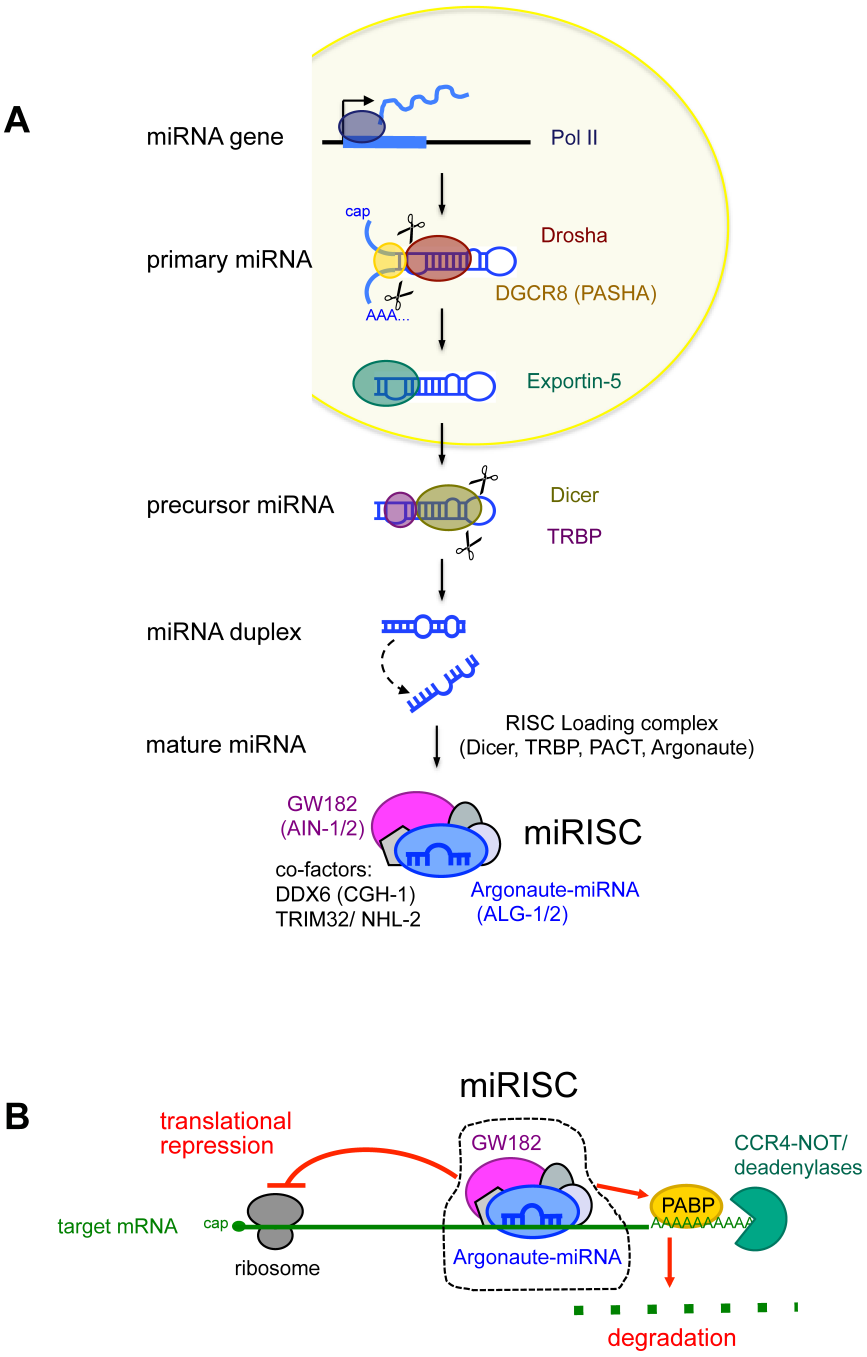

Table 1-1. miRNAs associated with common human disease.*

Disease	MiRNAs implicated	Reference
Cancer		
Breast	miR-23b/27b/24; miR-34a	[193], [194]
Chronic lymphoid leukemia	miR-15, miR-16	[33]
Colorectal & Pancreatic	miR-155	[195], [196]
Gastric	miR-25, miR-29, miR-93, miR-106b; mir-181c	[197], [198]
Liver	miR-7, miR-29b	[199], [199,200]
Lung	miR-34, miR-155	[201], [202]
Prostate	miR-143, miR-145	[203]
Multiple	*let-7* family	reviewed in [37]
Multiple	miR-21	[204], [12]
Neurodegenerative		
Alzheimer's	miR-9, miR-29b-1, miR-29a; miR-139-5p, miR-3470a	[205], [206]
Parkinson's	miR-7, miR-34b/34c, miR-133b, miR-153	reviewed in [207]
Immunity		
Multiple sclerosis	miR-34a, miR-145, miR-155, miR-326	[208], [209], [210]
Systemic lupus erythematosus	miR-146a	[211]
Type II diabetes	miR-29 family, miR-103, miR-107	[212], [213]
Cardiovascular		
Cardiac arrhythmia	miR-1	[214]
Cardiac hypertrophy	miR-150, miR-181b, miR-195	[215]
Cardiac fibrosis	miR-29	[216]
Metabolic & Cholesterol Regulation	miR-33, miR-133	[217],[218]
Viral		
HIV-1	miR-28, miR-125b, miR-150, miR-223, miR-382	[219]
Hepatitis C	miR-122	[220]
Influenza viruses	miR-21, miR-22, miR-30b-d, miR-200a, miR-223, *let-7f*	[221], [222]
** adapted from Ardekani and Naeini [223]*		

REFERENCES

1. Kloosterman WP, Plasterk RH (2006) The diverse functions of microRNAs in animal development and disease. Developmental cell 11: 441-450.
2. Stefani G, Slack FJ (2008) Small non-coding RNAs in animal development. Nature reviews Molecular cell biology 9: 219-230.
3. Alvarez-Garcia I, Miska EA (2005) MicroRNA functions in animal development and human disease. Development 132: 4653-4662.
4. Shi XB, Tepper CG, deVere White RW (2008) Cancerous miRNAs and their regulation. Cell cycle 7: 1529-1538.
5. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75: 843-854.
6. Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75: 855-862.
7. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, et al. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403: 901-906.
8. Pincus Z, Smith-Vikos T, Slack FJ (2011) MicroRNA predictors of longevity in Caenorhabditis elegans. PLoS genetics 7: e1002306.
9. Ashraf SI, McLoon AL, Sclarsic SM, Kunes S (2006) Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. Cell 124: 191-205.
10. Konopka W, Kiryk A, Novak M, Herwerth M, Parkitna JR, et al. (2010) MicroRNA loss enhances learning and memory in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 30: 14835-14842.
11. Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Puthanveettil SV, et al. (2009) Characterization of small RNAs in Aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. Neuron 63: 803-817.
12. Medina PP, Nolde M, Slack FJ (2010) OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature 467: 86-90.
13. Hill DA, Ivanovich J, Priest JR, Gurnett CA, Dehner LP, et al. (2009) DICER1 mutations in familial pleuropulmonary blastoma. Science 325: 965.
14. Kumar MS, Pester RE, Chen CY, Lane K, Chin C, et al. (2009) Dicer1 functions as a haploinsufficient tumor suppressor. Genes & development 23: 2700-2704.
15. Ono K, Kuwabara Y, Han J (2011) MicroRNAs and cardiovascular diseases. The FEBS journal 278: 1619-1633.
16. Zhu H, Shyh-Chang N, Segre AV, Shinoda G, Shah SP, et al. (2011) The Lin28/let-7 Axis Regulates Glucose Metabolism. Cell 147: 81-94.
17. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic acids research 34: D140-144.
18. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome research 19: 92-105.

19. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, et al. (2003) The microRNAs of Caenorhabditis elegans. Genes & development 17: 991-1008.
20. Nilsen TW (2007) Mechanisms of microRNA-mediated gene regulation in animal cells. Trends in genetics : TIG 23: 243-249.
21. Sempere LF, Dubrovsky EB, Dubrovskaya VA, Berger EM, Ambros V (2002) The expression of the let-7 small regulatory RNA is controlled by ecdysone during metamorphosis in Drosophila melanogaster. Developmental biology 244: 170-179.
22. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, et al. (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408: 86-89.
23. Liu S, Xia Q, Zhao P, Cheng T, Hong K, et al. (2007) Characterization and expression patterns of let-7 microRNA in the silkworm (Bombyx mori). BMC developmental biology 7: 88.
24. Christodoulou F, Raible F, Tomer R, Simakov O, Trachana K, et al. (2010) Ancient animal microRNAs and the evolution of tissue identity. Nature 463: 1084-1088.
25. Ninova M, Ronshaugen M, Griffiths-Jones S (2014) Conserved temporal patterns of microRNA expression in Drosophila support a developmental hourglass model. Genome biology and evolution 6: 2459-2467.
26. Roush S, Slack FJ (2008) The let-7 family of microRNAs. Trends in cell biology 18: 505-516.
27. Ruby JG, Jan C, Player C, Axtell MJ, Lee W, et al. (2006) Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell 127: 1193-1207.
28. Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP, et al. (2005) The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in Caenorhabditis elegans. Dev Cell 9: 403-414.
29. Caygill EE, Johnston LA (2008) Temporal regulation of metamorphic processes in Drosophila by the let-7 and miR-125 heterochronic microRNAs. Current biology : CB 18: 943-950.
30. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294: 853-858.
31. Sokol NS, Xu P, Jan YN, Ambros V (2008) Drosophila let-7 microRNA is required for remodeling of the neuromusculature during metamorphosis. Genes & development 22: 1591-1596.
32. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, et al. (2005) MicroRNA expression profiles classify human cancers. Nature 435: 834-838.
33. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, et al. (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proceedings of the National Academy of Sciences of the United States of America 99: 15524-15529.
34. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, et al. (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proceedings of the National Academy of Sciences of the United States of America 101: 2999-3004.

35. Calin GA, Croce CM (2006) MicroRNA signatures in human cancers. Nature reviews Cancer 6: 857-866.
36. Heravi-Moussavi A, Anglesio MS, Cheng SW, Senz J, Yang W, et al. (2012) Recurrent somatic DICER1 mutations in nonepithelial ovarian cancers. The New England journal of medicine 366: 234-242.
37. Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME (2010) The role of let-7 in cell differentiation and cancer. Endocrine-related cancer 17: F19-36.
38. Kasinski AL, Slack FJ (2011) Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. Nature reviews Cancer 11: 849-864.
39. Esquela-Kerscher A, Slack FJ (2006) Oncomirs - microRNAs with a role in cancer. Nature reviews Cancer 6: 259-269.
40. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, et al. (2007) The let-7 microRNA represses cell proliferation pathways in human cells. Cancer research 67: 7713-7722.
41. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, et al. (2005) RAS is regulated by the let-7 microRNA family. Cell 120: 635-647.
42. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T (2007) Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nature genetics 39: 673-677.
43. Lee YS, Dutta A (2007) The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes & development 21: 1025-1030.
44. Mayr C, Hemann MT, Bartel DP (2007) Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 315: 1576-1579.
45. Park SM, Shell S, Radjabi AR, Schickel R, Feig C, et al. (2007) Let-7 prevents early cancer progression by suppressing expression of the embryonic gene HMGA2. Cell Cycle 6: 2585-2590.
46. Sampson VB, Rong NH, Han J, Yang Q, Aris V, et al. (2007) MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer research 67: 9762-9770.
47. Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, et al. (2006) Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proceedings of the National Academy of Sciences of the United States of America 103: 7024-7029.
48. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, et al. (2005) A microRNA polycistron as a potential human oncogene. Nature 435: 828-833.
49. Mu P, Han YC, Betel D, Yao E, Squatrito M, et al. (2009) Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas. Genes & development 23: 2806-2811.
50. Pramanik D, Campbell NR, Karikari C, Chivukula R, Kent OA, et al. (2011) Restitution of tumor suppressor microRNAs using a systemic nanovector inhibits pancreatic cancer growth in mice. Molecular cancer therapeutics 10: 1470-1480.
51. Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, et al. (2011) Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. Molecular therapy : the journal of the American Society of Gene Therapy 19: 1116-1122.

52. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, et al. (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 137: 1005-1017.
53. Sulston JEaH, H. R. (1977) Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Developmental biology 56: 110-156.
54. Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode Caenorhabditis elegans. Developmental biology 100: 64-119.
55. Ecsedi M, Rausch M, Grosshans H (2015) The let-7 microRNA directs vulval development through a single target. Developmental cell 32: 335-344.
56. Lin YC, Hsieh LC, Kuo MW, Yu J, Kuo HH, et al. (2007) Human TRIM71 and its nematode homologue are targets of let-7 microRNA and its zebrafish orthologue is essential for development. Molecular biology and evolution 24: 2525-2534.
57. Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, et al. (2000) The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell 5: 659-669.
58. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, et al. (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 122: 553-563.
59. Lin SY, Johnson SM, Abraham M, Vella MC, Pasquinelli A, et al. (2003) The C elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. Developmental cell 4: 639-650.
60. Grosshans H, Johnson T, Reinert KL, Gerstein M, Slack FJ (2005) The temporal patterning microRNA let-7 regulates several transcription factors at the larval to adult transition in C. elegans. Developmental cell 8: 321-330.
61. Esquela-Kerscher A, Johnson SM, Bai L, Saito K, Partridge J, et al. (2005) Post-embryonic expression of C. elegans microRNAs belonging to the lin-4 and let-7 families in the hypodermis and the reproductive system. Developmental dynamics : an official publication of the American Association of Anatomists 234: 868-877.
62. Hunter SE, Finnegan EF, Zisoulis DG, Lovci MT, Melnik-Martinez KV, et al. (2013) Functional genomic analysis of the let-7 regulatory network in Caenorhabditis elegans. PLoS genetics 9: e1003353.
63. Chatterjee S, Grosshans H (2009) Active turnover modulates mature microRNA activity in Caenorhabditis elegans. Nature 461: 546-549.
64. Banerjee D, Kwok A, Lin SY, Slack FJ (2005) Developmental timing in C. elegans is regulated by kin-20 and tim-1, homologs of core circadian clock genes. Developmental cell 8: 287-295.
65. Rausch M, Ecsedi M, Bartake H, Mullner A, Grosshans H (2015) A genetic interactome of the let-7 microRNA in C. elegans. Developmental biology 401: 276-286.
66. Vasquez-Rifo A, Bosse GD, Rondeau EL, Jannot G, Dallaire A, et al. (2013) A new role for the GARP complex in microRNA-mediated gene regulation. PLoS genetics 9: e1003961.
67. Parry DH, Xu J, Ruvkun G (2007) A whole-genome RNAi Screen for C. elegans miRNA pathway genes. Curr Biol 17: 2013-2022.

68. Kim JK, Gabel HW, Kamath RS, Tewari M, Pasquinelli A, et al. (2005) Functional genomic analysis of RNA interference in C. elegans. Science 308: 1164-1167.
69. Tabach Y, Golan T, Hernandez-Hernandez A, Messer AR, Fukuda T, et al. (2013) Human disease locus discovery and mapping to molecular pathways through phylogenetic profiling. Molecular systems biology 9: 692.
70. Simon DJ, Madison JM, Conery AL, Thompson-Peer KL, Soskis M, et al. (2008) The microRNA miR-1 regulates a MEF-2-dependent retrograde signal at neuromuscular junctions. Cell 133: 903-915.
71. Hallam SJ, Jin Y (1998) lin-14 regulates the timing of synaptic remodelling in Caenorhabditis elegans. Nature 395: 78-82.
72. Johnston RJ, Hobert O (2003) A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature 426: 845-849.
73. Cai X, Hagedorn CH, Cullen BR (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10: 1957-1966.
74. Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002) MicroRNA maturation: stepwise processing and subcellular localization. The EMBO journal 21: 4663-4670.
75. Bracht J, Hunter S, Eachus R, Weeks P, Pasquinelli AE (2004) Trans-splicing and polyadenylation of let-7 microRNA primary transcripts. RNA 10: 1586-1594.
76. Lee Y, Ahn C, Han J, Choi H, Kim J, et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. Nature 425: 415-419.
77. Zeng Y, Yi R, Cullen BR (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proceedings of the National Academy of Sciences of the United States of America 100: 9779-9784.
78. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex. Nature 432: 231-235.
79. Han J, Lee Y, Yeom KH, Nam JW, Heo I, et al. (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125: 887-901.
80. Ruby JG, Jan CH, Bartel DP (2007) Intronic microRNA precursors that bypass Drosha processing. Nature 448: 83-86.
81. Bohnsack MT, Czaplinski K, Gorlich D (2004) Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA 10: 185-191.
82. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U (2004) Nuclear export of microRNA precursors. Science 303: 95-98.
83. Yi R, Qin Y, Macara IG, Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes & development 17: 3011-3016.
84. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, et al. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 106: 23-34.
85. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, et al. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293: 834-838.

86. Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, et al. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes & development 15: 2654-2659.
87. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, et al. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436: 740-744.
88. Forstemann K, Tomari Y, Du T, Vagin VV, Denli AM, et al. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS biology 3: e236.
89. Liu X, Park JK, Jiang F, Liu Y, McKearin D, et al. (2007) Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes. RNA 13: 2324-2329.
90. Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. Cell 115: 209-216.
91. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, et al. (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell 115: 199-208.
92. Lee HY, Zhou K, Smith AM, Noland CL, Doudna JA (2013) Differential roles of human Dicer-binding proteins TRBP and PACT in small RNA processing. Nucleic acids research 41: 6568-6576.
93. Maniataki E, Mourelatos Z (2005) A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes & development 19: 2979-2990.
94. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 123: 631-640.
95. Hutvagner G, Zamore PD (2002) A microRNA in a multiple-turnover RNAi enzyme complex. Science 297: 2056-2060.
96. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, et al. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes & development 16: 720-728.
97. Meister G (2013) Argonaute proteins: functional insights and emerging roles. Nature reviews Genetics 14: 447-459.
98. Bohmert K, Camus I, Bellini C, Bouchez D, Caboche M, et al. (1998) AGO1 defines a novel locus of Arabidopsis controlling leaf development. The EMBO journal 17: 170-180.
99. ArgoSearch. researchdata.museum.vic.gov.au/argo search/. accessed 06-01-2015.
100. Ding L, Spencer A, Morita K, Han M (2005) The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Mol Cell 19: 437-447.
101. Ding L, Han M (2007) GW182 family proteins are crucial for microRNA-mediated gene silencing. Trends Cell Biol 17: 411-416.
102. Eulalio A, Behm-Ansmant I, Izaurralde E (2007) P bodies: at the crossroads of post-transcriptional pathways. Nat Rev Mol Cell Biol 8: 9-22.
103. Parker R, Sheth U (2007) P bodies and the control of mRNA translation and degradation. Mol Cell 25: 635-646.
104. Zhang L, Ding L, Cheung TH, Dong MQ, Chen J, et al. (2007) Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Mol Cell 28: 598-613.

105. Jakymiw A, Lian S, Eystathioy T, Li S, Satoh M, et al. (2005) Disruption of GW bodies impairs mammalian RNA interference. Nature cell biology 7: 1267-1274.
106. Ding XC, Grosshans H (2009) Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins. EMBO J 28: 213-222.
107. Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, et al. (2009) Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol Cell. pp. 868-880.
108. Braun JE, Huntzinger E, Fauser M, Izaurralde E GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets. Mol Cell 44: 120-133.
109. Su H, Meng S, Lu Y, Trombly MI, Chen J, et al. Mammalian hyperplastic discs homolog EDD regulates miRNA-mediated gene silencing. Mol Cell 43: 97-109.
110. Funakoshi Y, Doi Y, Hosoda N, Uchida N, Osawa M, et al. (2007) Mechanism of mRNA deadenylation: evidence for a molecular interplay between translation termination factor eRF3 and mRNA deadenylases. Genes Dev 21: 3135-3148.
111. Rouya C, Siddiqui N, Morita M, Duchaine TF, Fabian MR, et al. (2014) Human DDX6 effects miRNA-mediated gene silencing via direct binding to CNOT1. RNA 20: 1398-1409.
112. Mathys H, Basquin J, Ozgur S, Czarnocki-Cieciura M, Bonneau F, et al. (2014) Structural and biochemical insights to the role of the CCR4-NOT complex and DDX6 ATPase in microRNA repression. Molecular cell 54: 751-765.
113. Chen Y, Boland A, Kuzuoglu-Ozturk D, Bawankar P, Loh B, et al. (2014) A DDX6-CNOT1 complex and W-binding pockets in CNOT9 reveal direct links between miRNA target recognition and silencing. Molecular cell 54: 737-750.
114. Hammell CM, Lubin I, Boag PR, Blackwell TK, Ambros V (2009) nhl-2 Modulates microRNA activity in Caenorhabditis elegans. Cell 136: 926-938.
115. Schwamborn JC, Berezikov E, Knoblich JA (2009) The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell 136: 913-925.
116. Caudy AA, Ketting RF, Hammond SM, Denli AM, Bathoorn AM, et al. (2003) A micrococcal nuclease homologue in RNAi effector complexes. Nature 425: 411-414.
117. Carthew RW, Sontheimer EJ (2009) Origins and Mechanisms of miRNAs and siRNAs. Cell 136: 642-655.
118. Wu L, Belasco JG (2008) Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. Mol Cell 29: 1-7.
119. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9: 102-114.
120. Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, et al. (2008) Widespread changes in protein synthesis induced by microRNAs. Nature 455: 58-63.
121. Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, et al. (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 309: 1573-1576.

122. Mathonnet G, Fabian MR, Svitkin YV, Parsyan A, Huck L, et al. (2007) MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science 317: 1764-1767.
123. Wakiyama M, Takimoto K, Ohara O, Yokoyama S (2007) Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes Dev 21: 1857-1862.
124. Kiriakidou M, Tan GS, Lamprinaki S, De Planell-Saguer M, Nelson PT, et al. (2007) An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell 129: 1141-1151.
125. Eulalio A, Huntzinger E, Nishihara T, Rehwinkel J, Fauser M, et al. (2009) Deadenylation is a widespread effect of miRNA regulation. RNA 15: 21-32.
126. Chekulaeva M, Mathys H, Zipprich JT, Attig J, Colic M, et al. (2011) miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. Nat Struct Mol Biol. pp. 1218-1226.
127. Djuranovic S, Nahvi A, Green R miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science 336: 237-240.
128. Ingolia NT, Ghaemmaghami S, Newman JRS, Weissman JS (2009) Genome-Wide Analysis in Vivo of Translation with Nucleotide Resolution Using Ribosome Profiling. Science. pp. 218-223.
129. Guo H, Ingolia NT, Weissman JS, Bartel DP (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. pp. 835-840.
130. Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, et al. (2006) mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev 20: 1885-1898.
131. Eulalio A, Rehwinkel J, Stricker M, Huntzinger E, Yang SF, et al. (2007) Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. Genes Dev 21: 2558-2570.
132. Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, et al. (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312: 75-79.
133. Wu L, Fan J, Belasco JG (2006) MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A 103: 4034-4039.
134. Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, et al. (2009) Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol Cell 35: 868-880.
135. Zekri L, Huntzinger E, Heimstadt S, Izaurralde E (2009) The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. Mol Cell Biol 29: 6220-6231.
136. Krol J, Loedige I, Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. Nature reviews Genetics 11: 597-610.
137. Geiss-Friedlander R, Melchior F (2007) Concepts in sumoylation: a decade on. Nature reviews Molecular cell biology 8: 947-956.
138. Morrison RS, Kinoshita Y, Johnson MD, Uo T, Ho JT, et al. (2002) Proteomic analysis in the neurosciences. Molecular & cellular proteomics : MCP 1: 553-560.

139. Wilczynska A, Bushell M (2015) The complexity of miRNA-mediated repression. Cell death and differentiation 22: 22-33.
140. Rudel S, Wang Y, Lenobel R, Korner R, Hsiao HH, et al. (2011) Phosphorylation of human Argonaute proteins affects small RNA binding. Nucleic acids research 39: 2330-2343.
141. Zeng Y, Sankala H, Zhang X, Graves PR (2008) Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. The Biochemical journal 413: 429-436.
142. Horman SR, Janas MM, Litterst C, Wang B, MacRae IJ, et al. (2013) Akt-mediated phosphorylation of argonaute 2 downregulates cleavage and upregulates translational repression of MicroRNA targets. Molecular cell 50: 356-367.
143. Qi HH, Ongusaha PP, Myllyharju J, Cheng D, Pakkanen O, et al. (2008) Prolyl 4-hydroxylation regulates Argonaute 2 stability. Nature 455: 421-424.
144. Wu C, So J, Davis-Dusenbery BN, Qi HH, Bloch DB, et al. (2011) Hypoxia potentiates microRNA-mediated gene silencing through posttranslational modification of Argonaute2. Molecular and cellular biology 31: 4760-4774.
145. Rybak A, Fuchs H, Hadian K, Smirnova L, Wulczyn EA, et al. (2009) The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. Nature cell biology 11: 1411-1420.
146. Leung AK, Vyas S, Rood JE, Bhutkar A, Sharp PA, et al. (2011) Poly(ADP-ribose) regulates stress responses and microRNA activity in the cytoplasm. Molecular cell 42: 489-499.
147. Huang KL, Chadee AB, Chen CY, Zhang Y, Shyu AB (2013) Phosphorylation at intrinsically disordered regions of PAM2 motif-containing proteins modulates their interactions with PABPC1 and influences mRNA fate. RNA 19: 295-305.
148. Gherzi R, Lee KY, Briata P, Wegmuller D, Moroni C, et al. (2004) A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. Molecular cell 14: 571-583.
149. Xu N, Chen CY, Shyu AB (2001) Versatile role for hnRNP D isoforms in the differential regulation of cytoplasmic mRNA turnover. Molecular and cellular biology 21: 6960-6971.
150. Lai WS, Kennington EA, Blackshear PJ (2003) Tristetraprolin and its family members can promote the cell-free deadenylation of AU-rich element-containing mRNAs by poly(A) ribonuclease. Molecular and cellular biology 23: 3798-3812.
151. Bolognani F, Perrone-Bizzozero NI (2008) RNA-protein interactions and control of mRNA stability in neurons. Journal of neuroscience research 86: 481-489.
152. Peng SS, Chen CY, Xu N, Shyu AB (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. The EMBO journal 17: 3461-3470.
153. Spassov DS, Jurecic R (2003) The PUF family of RNA-binding proteins: does evolutionarily conserved structure equal conserved function? IUBMB life 55: 359-366.
154. Keene JD (2007) RNA regulons: coordination of post-transcriptional events. Nature reviews Genetics 8: 533-543.
155. Cook KB, Kazan H, Zuberi K, Morris Q, Hughes TR (2011) RBPDB: a database of RNA-binding specificities. Nucleic acids research 39: D301-308.

156. Kedde M, Strasser MJ, Boldajipour B, Oude Vrielink JA, Slanchev K, et al. (2007) RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. Cell 131: 1273-1286.
157. Nolde MJ, Saka N, Reinert KL, Slack FJ (2007) The Caenorhabditis elegans pumilio homolog, puf-9, is required for the 3'UTR-mediated repression of the let-7 microRNA target gene, hbl-1. Developmental biology 305: 551-563.
158. Galgano A, Forrer M, Jaskiewicz L, Kanitz A, Zavolan M, et al. (2008) Comparative analysis of mRNA targets for human PUF-family proteins suggests extensive interaction with the miRNA regulatory system. PloS one 3: e3164.
159. Srikantan S, Tominaga K, Gorospe M (2012) Functional interplay between RNA-binding protein HuR and microRNAs. Current protein & peptide science 13: 372-379.
160. Mukherjee N, Corcoran DL, Nusbaum JD, Reid DW, Georgiev S, et al. (2011) Integrative regulatory mapping indicates that the RNA-binding protein HuR couples pre-mRNA processing and mRNA stability. Molecular cell 43: 327-339.
161. Lebedeva S, Jens M, Theil K, Schwanhausser B, Selbach M, et al. (2011) Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR. Molecular cell 43: 340-352.
162. Kim HH, Kuwano Y, Srikantan S, Lee EK, Martindale JL, et al. (2009) HuR recruits let-7/RISC to repress c-Myc expression. Genes & development 23: 1743-1748.
163. Glorian V, Maillot G, Poles S, Iacovoni JS, Favre G, et al. (2011) HuR-dependent loading of miRNA RISC to the mRNA encoding the Ras-related small GTPase RhoB controls its translation during UV-induced apoptosis. Cell death and differentiation 18: 1692-1701.
164. Chang N, Yi J, Guo G, Liu X, Shang Y, et al. (2010) HuR uses AUF1 as a cofactor to promote p16INK4 mRNA decay. Molecular and cellular biology 30: 3875-3886.
165. Xue Y, Ouyang K, Huang J, Zhou Y, Ouyang H, et al. (2013) Direct conversion of fibroblasts to neurons by reprogramming PTB-regulated microRNA circuits. Cell 152: 82-96.
166. Kenny PJ, Zhou H, Kim M, Skariah G, Khetani RS, et al. (2014) MOV10 and FMRP regulate AGO2 association with microRNA recognition elements. Cell reports 9: 1729-1741.
167. Litchfield DW (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. The Biochemical journal 369: 1-15.
168. Pinna LA (2002) Protein kinase CK2: a challenge to canons. Journal of cell science 115: 3873-3878.
169. Graham KC, Litchfield DW (2000) The regulatory beta subunit of protein kinase CK2 mediates formation of tetrameric CK2 complexes. The Journal of biological chemistry 275: 5003-5010.
170. Allende JE, Allende CC (1995) Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 9: 313-323.
171. Ahmed K, Gerber DA, Cochet C (2002) Joining the cell survival squad: an emerging role for protein kinase CK2. Trends in cell biology 12: 226-230.

172. Pyerin W, Ackermann K (2003) The genes encoding human protein kinase CK2 and their functional links. Progress in nucleic acid research and molecular biology 74: 239-273.
173. Meggio F, Pinna LA (2003) One-thousand-and-one substrates of protein kinase CK2? FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17: 349-368.
174. Padmanabha R, Chen-Wu JL, Hanna DE, Glover CV (1990) Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in Saccharomyces cerevisiae. Molecular and cellular biology 10: 4089-4099.
175. Buchou T, Vernet M, Blond O, Jensen HH, Pointu H, et al. (2003) Disruption of the regulatory beta subunit of protein kinase CK2 in mice leads to a cell-autonomous defect and early embryonic lethality. Molecular and cellular biology 23: 908-915.
176. Wang X, Gupta P, Fairbanks J, Hansen D (2014) Protein kinase CK2 both promotes robust proliferation and inhibits the proliferative fate in the C. elegans germ line. Developmental biology 392: 26-41.
177. Hu J, Bae YK, Knobel KM, Barr MM (2006) Casein kinase II and calcineurin modulate TRPP function and ciliary localization. Molecular biology of the cell 17: 2200-2211.
178. Niefind K, Raaf J, Issinger OG (2009) Protein kinase CK2 in health and disease: Protein kinase CK2: from structures to insights. Cell Mol Life Sci 66: 1800-1816.
179. WormBase (ws248). wormbase.org. accessed 06-04-2015.
180. Eliseeva IA, Kim ER, Guryanov SG, Ovchinnikov LP, Lyabin DN (2011) Y-box-binding protein 1 (YB-1) and its functions. Biochemistry Biokhimiia 76: 1402-1433.
181. Medvedev S, Pan H, Schultz RM (2011) Absence of MSY2 in mouse oocytes perturbs oocyte growth and maturation, RNA stability, and the transcriptome. Biology of reproduction 85: 575-583.
182. Yang J, Medvedev S, Yu J, Schultz RM, Hecht NB (2006) Deletion of the DNA/RNA-binding protein MSY2 leads to post-meiotic arrest. Molecular and cellular endocrinology 250: 20-24.
183. Bouvet P, Wolffe AP (1994) A role for transcription and FRGY2 in masking maternal mRNA within Xenopus oocytes. Cell 77: 931-941.
184. Mansfield JH, Wilhelm JE, Hazelrigg T (2002) Ypsilon Schachtel, a Drosophila Y-box protein, acts antagonistically to Orb in the oskar mRNA localization and translation pathway. Development 129: 197-209.
185. Kumari P, Gilligan PC, Lim S, Tran LD, Winkler S, et al. (2013) An essential role for maternal control of Nodal signaling. eLife 2: e00683.
186. Arnold A, Rahman MM, Lee MC, Muehlhaeusser S, Katic I, et al. (2014) Functional characterization of C. elegans Y-box-binding proteins reveals tissue-specific functions and a critical role in the formation of polysomes. Nucleic acids research 42: 13353-13369.
187. Kohno K, Izumi H, Uchiumi T, Ashizuka M, Kuwano M (2003) The pleiotropic functions of the Y-box-binding protein, YB-1. BioEssays : news and reviews in molecular, cellular and developmental biology 25: 691-698.
188. Kuwano M, Oda Y, Izumi H, Yang SJ, Uchiumi T, et al. (2004) The role of nuclear Y-box binding protein 1 as a global marker in drug resistance. Molecular cancer therapeutics 3: 1485-1492.

189. Matsumoto K, Bay BH (2005) Significance of the Y-box proteins in human cancers. Journal of molecular and genetic medicine : an international journal of biomedical research 1: 11-17.
190. Skabkin MA, Kiselyova OI, Chernov KG, Sorokin AV, Dubrovin EV, et al. (2004) Structural organization of mRNA complexes with major core mRNP protein YB-1. Nucleic acids research 32: 5621-5635.
191. Evdokimova V, Ruzanov P, Anglesio MS, Sorokin AV, Ovchinnikov LP, et al. (2006) Akt-mediated YB-1 phosphorylation activates translation of silent mRNA species. Molecular and cellular biology 26: 277-292.
192. Soop T, Nashchekin D, Zhao J, Sun X, Alzhanova-Ericsson AT, et al. (2003) A p50-like Y-box protein with a putative translational role becomes associated with pre-mRNA concomitant with transcription. Journal of cell science 116: 1493-1503.
193. Ell B, Qiu Q, Wei Y, Mercatali L, Ibrahim T, et al. (2014) The microRNA-23b/27b/24 cluster promotes breast cancer lung metastasis by targeting metastasis-suppressive gene prosaposin. The Journal of biological chemistry 289: 21888-21895.
194. Krzeszinski JY, Wei W, Huynh H, Jin Z, Wang X, et al. (2014) miR-34a blocks osteoporosis and bone metastasis by inhibiting osteoclastogenesis and Tgif2. Nature 512: 431-435.
195. Valeri N, Gasparini P, Fabbri M, Braconi C, Veronese A, et al. (2010) Modulation of mismatch repair and genomic stability by miR-155. Proceedings of the National Academy of Sciences of the United States of America 107: 6982-6987.
196. Berger KI, Goldring RM, Oppenheimer BW (2015) Point: Should Oscillometry be used to Screen for Airway Disease: Yes. Chest.
197. Hashimoto Y, Akiyama Y, Otsubo T, Shimada S, Yuasa Y (2010) Involvement of epigenetically silenced microRNA-181c in gastric carcinogenesis. Carcinogenesis 31: 777-784.
198. Espinosa-Parrilla Y, Munoz X, Bonet C, Garcia N, Vencesla A, et al. (2014) Genetic association of gastric cancer with miRNA clusters including the cancer-related genes MIR29, MIR25, MIR93 and MIR106: results from the EPIC-EURGAST study. International journal of cancer Journal international du cancer 135: 2065-2076.
199. Lin LL, Wang W, Hu Z, Wang LW, Chang J, et al. (2014) Negative feedback of miR-29 family TET1 involves in hepatocellular cancer. Medical oncology 31: 291.
200. Kim BK, Jo HS, Lee HJ (2015) Study on the factors related with intention of cancer screening among Korean residents: application of information-motivation-behavioral skills model. Asia-Pacific journal of public health / Asia-Pacific Academic Consortium for Public Health 27: NP2133-2143.
201. Kumar MS, Armenteros-Monterroso E, East P, Chakravorty P, Matthews N, et al. (2014) HMGA2 functions as a competing endogenous RNA to promote lung cancer progression. Nature 505: 212-217.
202. Stahlhut C, Slack FJ (2015) Combinatorial Action of MicroRNAs let-7 and miR-34 Effectively Synergizes with Erlotinib to Suppress Non-small Cell Lung Cancer Cell Proliferation. Cell cycle: 0.

203. Peng X, Guo W, Liu T, Wang X, Tu X, et al. (2011) Identification of miRs-143 and -145 that is associated with bone metastasis of prostate cancer and involved in the regulation of EMT. PloS one 6: e20341.
204. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, et al. (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. Proceedings of the National Academy of Sciences of the United States of America 103: 2257-2261.
205. Nunez-Iglesias J, Liu CC, Morgan TE, Finch CE, Zhou XJ (2010) Joint genome-wide profiling of miRNA and mRNA expression in Alzheimer's disease cortex reveals altered miRNA regulation. PloS one 5: e8898.
206. Noh H, Park C, Park S, Lee YS, Cho SY, et al. (2014) Prediction of miRNA-mRNA associations in Alzheimer's disease mice using network topology. BMC genomics 15: 644.
207. Mouradian MM (2012) MicroRNAs in Parkinson's disease. Neurobiology of disease 46: 279-284.
208. Keller A, Leidinger P, Lange J, Borries A, Schroers H, et al. (2009) Multiple sclerosis: microRNA expression profiles accurately differentiate patients with relapsing-remitting disease from healthy controls. PloS one 4: e7440.
209. Junker A, Krumbholz M, Eisele S, Mohan H, Augstein F, et al. (2009) MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. Brain : a journal of neurology 132: 3342-3352.
210. Du C, Liu C, Kang J, Zhao G, Ye Z, et al. (2009) MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. Nature immunology 10: 1252-1259.
211. Lofgren SE, Frostegard J, Truedsson L, Pons-Estel BA, D'Alfonso S, et al. (2012) Genetic association of miRNA-146a with systemic lupus erythematosus in Europeans through decreased expression of the gene. Genes and immunity 13: 268-274.
212. He A, Zhu L, Gupta N, Chang Y, Fang F (2007) Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. Molecular endocrinology 21: 2785-2794.
213. Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, et al. (2011) MicroRNAs 103 and 107 regulate insulin sensitivity. Nature 474: 649-653.
214. Yang B, Lin H, Xiao J, Lu Y, Luo X, et al. (2007) The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nature medicine 13: 486-491.
215. van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, et al. (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proceedings of the National Academy of Sciences of the United States of America 103: 18255-18260.
216. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, et al. (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proceedings of the National Academy of Sciences of the United States of America 105: 13027-13032.

217. Marquart TJ, Allen RM, Ory DS, Baldan A (2010) miR-33 links SREBP-2 induction to repression of sterol transporters. Proceedings of the National Academy of Sciences of the United States of America 107: 12228-12232.
218. Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, et al. (2010) MiR-33 contributes to the regulation of cholesterol homeostasis. Science 328: 1570-1573.
219. Huang J, Wang F, Argyris E, Chen K, Liang Z, et al. (2007) Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. Nature medicine 13: 1241-1247.
220. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P (2005) Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309: 1577-1581.
221. Li Y, Li J, Belisle S, Baskin CR, Tumpey TM, et al. (2011) Differential microRNA expression and virulence of avian, 1918 reassortant, and reconstructed 1918 influenza A viruses. Virology 421: 105-113.
222. Li Y, Chan EY, Li J, Ni C, Peng X, et al. (2010) MicroRNA expression and virulence in pandemic influenza virus-infected mice. Journal of virology 84: 3023-3032.
223. Ardekani AM, Naeini MM (2010) The Role of MicroRNAs in Human Diseases. Avicenna journal of medical biotechnology 2: 161-179.

CHAPTER 2

Casein kinase II promotes target silencing by miRISC through direct phosphorylation of the DEAD-box helicase CGH-1 [§]

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[§] manuscript was prepared by AFA and JKK and is currently in the final stages of revision at *Proceedings of the National Academy of Sciences.*

AUTHOR CONTRIBUTIONS

AFA, TH, XK, and JKK designed experiments. JM, PGT, and JRY performed the mass spectrometry analyses. MAF performed computational analyses. AFA, VK, TH, XK and EM performed all other experiments. AFA, TH, and JKK wrote the manuscript.

ABSTRACT

MicroRNAs (miRNAs) play essential, conserved roles in diverse developmental processes through association with the microRNA-Induced Silencing Complex (miRISC). While fundamental insights into the mechanistic framework of miRNA biogenesis and target gene silencing have been established, post-translational modifications that affect miRISC function are less well understood. Here we report that

the conserved serine/threonine kinase, casein kinase II (CK2), promotes miRISC function in *Caenorhabditis elegans*. CK2 inactivation results in developmental timing defects that phenocopy loss of miRISC cofactors and enhances the loss of miRNA function in diverse cellular contexts. While CK2 is dispensable for miRNA biogenesis and the stability of miRISC cofactors, it is required for efficient miRISC target mRNA binding and silencing. Importantly, we identify the conserved DEAD-box RNA helicase, CGH-1/DDX6, as a key CK2 substrate within miRISC and demonstrate phosphorylation of a conserved N-terminal serine is required for CGH-1 function in the miRNA pathway.

SIGNIFICANCE STATEMENT

MicroRNAs are critical regulators of diverse biological processes. Despite rapid advances in understanding microRNA biogenesis and function, a gap remains in our knowledge of how microRNA effector complex activity (miRISC) is modulated. Specifically, the importance of post-translational protein modifications in controlling miRISC activity remains largely unexplored. Here, we characterize a novel role for the conserved serine/threonine kinase, casein kinase II (CK2), in promoting the miRNA pathway in *C. elegans*. Notably, we establish the requirement of CK2 for miRNA function and provide mechanistic evidence that loss of CK2 compromises miRISC binding to mRNA targets. Furthermore, we identify that the miRISC cofactor and DEAD-box RNA helicase, CGH-1/DDX6, is phosphorylated by CK2 at a conserved residue, which is required for CGH-1-mediated miRNA function.

INTRODUCTION

Since the discovery of *lin-4* and *let-7* in *C. elegans*, miRNAs have emerged as an evolutionarily conserved superfamily of small endogenous RNAs critical for post-transcriptional gene regulation (Lee et al., 1993; Reinhart et al., 2000). MiRNAs regulate diverse biological processes including animal development, cell differentiation, apoptosis, and metabolism [1,2,3]. To date, at least 250 miRNAs have been identified in *C. elegans* and almost ten times as many in human [4]. Furthermore, miRNAs are predicted to target as much as half of the transcriptome, further underscoring their central role in post-transcriptional gene silencing mechanisms [5].

MiRNAs are typically transcribed as long primary transcripts that are processed sequentially by the RNase III enzymes Drosha and Dicer to produce a ~70 nt precursor hairpin and a ~22 nt RNA duplex, respectively [6,7]. One strand of the duplex is selectively loaded as the mature miRNA into an Argonaute (Ago) family protein that forms the core of miRISC in conjunction with a GW182 family protein [6,7]. Partial base pairing of the miRNA with complementary sites predominantly located in the 3'UTR of target mRNAs leads to transcript destabilization, translational repression, or both [3,8]. Through a combination of genetic and biochemical approaches, conserved miRISC factors have been identified in *C. elegans* including Argonaute-like genes ALG-1 and ALG-2, orthologs of human Ago2 [9]. Mutation of *alg-1* results in pleiotropic phenotypes, consistent with loss of miRNA regulation in diverse biological pathways [9,10]. In contrast, *alg-2* mutants are superficially wild-type, suggesting a partially redundant role in miRNA-mediated processes [10]. ALG-1 physically interacts with AIN-1 and AIN-2 (ALG-1 interacting protein), functionally redundant orthologs of human GW182 [11,12].

AIN-1/2 are required for the localization of ALG-1 to P-bodies, major cytoplasmic centers for mRNA catabolism and storage. Thus, AIN-1/2 function as a molecular link between target-bound miRISC and downstream machinery required for translational repression and degradation of target mRNAs [11,12].

Activity of miRISC is facilitated by conserved cofactors that include the DEAD-box RNA helicase CGH-1 and the TRIM-NHL ubiquitin ligase NHL-2. CGH-1 and its homologs are broadly involved in regulating mRNA stability and translation during development and differentiation in both somatic and germ cells [13,14]. In *C. elegans, cgh-1* and *nhl-2* are required for effective miRISC target silencing [15]. The human ortholog of CGH-1, DDX6, has been shown to promote miRISC by interaction with the CCR4-NOT deadenylase complex and mRNA degradation machinery [16,17,18]. The *C. elegans* miRISC also contains the fly Vasa Intronic Gene ortholog, VIG-1, and the Tudor domain protein, TSN-1, that have both been shown to be required for silencing of a *let-7* target 3'UTR reporter [19].

Post-translational modifications (PTMs) have recently emerged as a potential mechanism for directly regulating miRISC activity [20]. To date, a limited number of studies have addressed the functional role of PTMs of miRNA pathway machinery. In particular, several PTMs to Argonaute have been characterized [21,22,23,24,25,26,27,28,29,30]. However, little is known about the identity and functional significance PTMs of other components of miRISC.

Previously, we reported the identification of several signaling kinases including the conserved, constitutive serine/threonine kinase, casein kinase II (CK2), in a genome-wide RNAi screen for candidate factors involved in small RNA-mediated

silencing in *C. elegans* [31,32]. In this study, we characterize the role of CK2 as a positive regulator of miRISC activity in *C. elegans*. We provide evidence that CK2 functions downstream of miRNA biogenesis and is necessary for efficient miRISC binding to target mRNAs. Furthermore, we demonstrate that CK2 is required for phosphorylation of the miRISC cofactor CGH-1, which was previously implicated in facilitating miRISC-target interactions [15]. Our data indicate that CK2 phosphorylation of CGH-1 at a conserved serine residue within an N-terminal CK2 motif is required for CGH-1 function in developmental pathways regulated by miRNAs.

RESULTS

The CK2 holoenzyme consists of two catalytic α subunits and two regulatory β subunits in a tetrameric α2β2 configuration [33]. In *C. elegans*, *kin-3* and *kin-10* encode the catalytic and regulatory subunits of CK2, respectively [34]. In addition to identifying *kin-10* in a genome-wide screen for factors required for RNAi [31], we identified both CK2 subunits among factors that co-immunopurified with the GW182 homolog, AIN-1, by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). In addition to other core miRISC factors (ALG-1, ALG-2, and CGH-1), we recovered one KIN-3 peptide (KVLGTDELYEYIARY; 3.6% sequence coverage) and two KIN-10 peptides (RGNEFFCEVDEEYIQDRF, RFNLTGLNEQVPKY; 12% sequence coverage), suggesting that CK2 physically interacts with miRISC.

CK2 regulates miRNA-dependent developmental timing

To explore the possibility that CK2 regulates miRNA-dependent developmental timing,

we analyzed animals depleted of *kin-3* or *kin-10* for phenotypes associated with compromised miRISC function [9,11,15]. During post-embryonic development, *C. elegans* proceeds through four larval stages (L1-L4) before emerging as an adult. Loss of core miRISC factors results in reiteration of larval developmental patterns and failure or delay in the initiation of adult-specific programs. For example, formation of cuticle protrusions, termed alae, along the left and right sides of the adult animal requires the *let-7* family of miRNAs [2,35]. We investigated if CK2 was required for the timing and proper formation of adult alae. Because CK2 is essential for viability, we used *kin-3* and *kin-10* RNAi (hereafter referred to as CK2 RNAi) to attenuate its expression (Fig 2-S1 A,B). While wild-type animals on empty vector control RNAi display no alae defects, RNAi of CK2 results in penetrant adult alae defects similar to those observed in RNAi of *alg-1,* which encodes the primary Argonaute protein in miRISC (Fig 2-1A). Similarly, seam cells are generated through stem cell-like divisions during larval transitions, terminally differentiating in the adult to yield 16 laterally distributed seam cells on each side of the body [36] that can be visualized using a seam cell-specific GFP reporter [37]. Whereas wild-type animals invariably have 16 seam cells, animals subjected to CK2 RNAi exhibit significantly increased numbers of seam cells, similar to animals depleted of miRISC components ALG-1, AIN-1, or NHL-2 (Fig 2-1B). These data indicate that CK2 is required for appropriate timing of seam cell divisions. Finally, we examined the timing of expression of a GFP reporter for the adult-specific collagen, COL-19, whose expression is controlled by miRNAs that regulate the heterochronic pathway [38] [39]. While only ~5% of adult animals on empty vector control RNAi display reduced P*col-19::gfp* expression in hypodermal cells at the adult stage, 20-35% of adult animals on

CK2 RNAi exhibit reduced GFP reporter expression, comparable to RNAi of core miRISC factors (Fig 2-S2A). Taken together, these results indicate that, like miRISC components, CK2 is required for miRNA-mediated adult hypodermal remodeling during the larval-to-adult transition.

CK2 promotes *let-7* family and miRISC function

To define further the role of CK2 in the miRNA pathway, we examined genetic interactions between CK2 and *let-7* family miRNAs and miRISC components. Mild phenotypes elicited by hypomorphic mutations in miRNA pathway genes create sensitized genetic backgrounds that can be enhanced by mutations in other genes required for the pathway. Therefore we examined if loss of *kin-3* or *kin-10* could enhance phenotypes associated with hypomorphic mutations in genes of the miRNA pathway.

In the transition period between each larval stage, *C. elegans* proceed through a quiescent state called lethargus, where animals cease pharyngeal pumping and reduce locomotion prior to molting. In retarded heterochronic mutants, lethargus is reiterated at the adult stage [2,35,40]. A mutant for *mir-48,* a member of the *let-7* family of miRNAs, exhibits a low level of adult lethargus that is exacerbated by deleting its paralog, *mir-84* [35] (Fig 2-1C). In the *mir-48*(*n4097*) sensitized genetic background, adult lethargus is significantly enhanced by CK2 RNAi at levels comparable to RNAi of miRISC components (Fig 2-1C). Likewise, loss of *let-7* activity leads to a rupturing (Rup) phenotype, in which animals herniate through the vulva and subsequently die [2]. Two hypomorphic *let-7* alleles (*mg279* and *n2853*) exhibit a mild Rup phenotype, which is

enhanced by CK2 RNAi at levels comparable to RNAi of miRISC components (Fig 2-1D). *let-7* family mutants, *mir-48*(*n4097*) and the *mir-48*(*n4097*); *mir-84*(*n4037*) double mutant [35], as well as core miRISC factor mutants, *alg-1*(*tm369*) and *ain-1*(*ku322*) [9,12] also display Rup phenotypes that are exacerbated by CK2 RNAi (Fig 2-1D). Together these data suggest loss of CK2 enhances mutant phenotypes of the *let-7* family of miRNAs.

To rule out the possibility of off-target effects of RNAi, we confirmed enhancement of *let-7* mutant phenotypes in *kin-10* deletion mutants. CK2 is essential for *C. elegans* development: *kin-3* (-/-) null mutants arrest as L3 larvae and *kin-10* (-/-) as L4 larvae. Consequently, CK2 null mutants are maintained in a genetically balanced background. Since the Rup phenotype can only be assessed in animals that reach the L4 stage, we examined two deletion mutants of *kin-10* (*ok1751* and *ok2031*) in the sensitized *let-7(mg279)* background. Consistent with results using *kin-10* RNAi, we observe an enhanced Rup phenotype in *kin-10* (-/-) relative to the balanced *kin-10* (+/-) siblings for both *kin-10* alleles (Fig 2-1E). Importantly, these data indicate that the *kin-10* genetic mutant recapitulates the phenotypes observed by *kin-10* RNAi.

The Rupture phenotype (Rup) of *let-7* family mutants can be partially suppressed by concurrent depletion of their mRNA targets [35,41]. Consistent with the hypothesis that CK2 promotes miRISC-mediated target silencing, Rup enhancement by CK2 RNAi in the *let-7* and *miR-48* mutants is suppressed by hypomorphic mutations in their respective targets, *lin-41* and *hbl-1* (Fig 2-1F,G). miR-84 also regulates vulval precursor cell (VPC) fate specification by attenuating the expression of its target *let-60/RAS* [42,43]. Failure to repress LET-60 activity in specific VPCs leads to vulval cell

misspecification and a multi-vulva phenotype (Muv) [42]. CK2 RNAi enhances Muv in a mild gain-of-function *let-60(ga89)* allele [44] (Fig 2-1H). Taken together, these data indicate that CK2 genetically interacts with the *let-7* family of miRNAs to promote miRISC function in a target-dependent manner.

CK2 is required for the function of *lsy-6* and miR-35 family miRNAs.

Since CK2 is broadly expressed (Fig 2-S2B), we tested whether it is required for the function of non-*let-7* family miRNAs. The miRNA *lsy-6* functions to specify the fate of ASEL, one of two morphologically similar, bilaterally symmetric ASE neurons (ASEL and ASER), which can be monitored by expression of an ASEL-specific P*lim-6*::*gfp* reporter [45]. Because many neurons in *C. elegans* are refractory to RNAi [46], we performed RNAi experiments in an *nre-1* mutant background, which potentiates RNAi-mediated gene silencing in neurons [47]. CK2 RNAi enhances the penetrance of ASEL mis-specification in the *lsy-6*(*ot150*) hypomorph [48] to the same extent as *alg-1* RNAi (Fig 2-1I), indicating that CK2 promotes *lsy-6* function.

The miR-35 family of miRNAs (miR-35-42) is expressed during embryogenesis and functions redundantly to control embryonic development [49]. Deletion of *miR-35-41* leads to semi-penetrant, temperature-sensitive late embryonic lethality [49]. At the permissive temperature (15°C), ~10% of embryos display this phenotype, while the remaining 90% develop normally. Depletion of CK2 or the gene encoding the core miRISC factor *nhl-2* significantly increases embryonic lethality, consistent with the requirement of CK2 for the function of the miR-35 family of miRNAs (Fig 2-1J).

Collectively, our results are consistent with CK2 broadly promoting miRNA function to mediate a range of biological processes in multiple *C. elegans* tissues.

CK2 is required for miRNA target silencing

Since KIN-3 and KIN-10 depletion enhances multiple miRNA mutant phenotypes, we sought to determine if these CK2-dependent physiological defects could be explained by miRNA target de-repression. We examined the endogenous, developmentally regulated targets of both *let-7* family and *lin-4* miRNAs (Fig 2-2A). In *C. elegans*, *let-7* triggers the rapid degradation of *lin-41* mRNA at the L4-to-adult transition [50,51]. *lin-41* encodes a TRIM/RBCC family protein, homologous to human TRIM71 [52], that negatively regulates expression of LIN-29, a transcription factor required for adult cell fate specification [50]. Depletion of *alg-1* leads to significant de-repression of *lin-41* mRNA compared to empty vector RNAi (Fig 2-2B). Similarly, knockdown of CK2 also significantly de-represses *lin-41* levels by 1.5 to 2 fold (Fig 2-2B, left panel). De-repression is also observed for the *let-7* family target *daf-12,* which encodes a nuclear steroid hormone receptor that integrates environmental signals and developmental timing [51,53] (Fig 2-2B, right panel).

To determine if de-repression of target mRNAs by CK2 RNAi results in elevated target mRNA translation, we examined endogenous protein levels of LIN-14 upon CK2 RNAi. The early-acting heterochronic miRNA, *lin-4*, targets and silences *lin-14* mRNA during the L1-to-L2 larval transition [1,54], resulting in a commensurate decrease in LIN-14 protein. In animals fed empty vector control RNAi, the LIN-14 protein is robustly expressed during the L1 stage (4 hr) and dramatically decreases by the L2 stage (Fig 2-

2C; 20 hr). In contrast, when animals are subjected to CK2 RNAi, LIN-14 levels remain high during the L1 to L2 transition to a similar degree, or possibly higher, than animals on *alg-1* RNAi (Fig 2-2C; 16-20 hr time points). The elevated LIN-14 expression is not due to any dramatic developmental delay caused by CK2 or *alg-1* RNAi during this timeframe based on measurements of animal growth (Fig 2-S3).

Next we examined miR-1-dependent silencing of *mef-2*, which encodes a muscle-specific transcription factor [55], using a 3'UTR GFP reporter driven by the muscle-specific *myo-3* promoter (Fig 2-2D). Depletion of *kin-3, kin-10*, or *alg-1* significantly increases the intensity of *mef-2* reporter expression compared to empty vector RNAi (Fig 2-2D,E). To test the miR-1 dependence of target reporter expression, we scrambled the two miR-1 binding sites in the *mef-2* 3'UTR. Consistent with a miR-1-dependent effect, no significant difference in GFP expression was detected in the scrambled reporter with CK2 or *alg-1* RNAi versus empty vector control (Fig 2-2D,E). Taken together, our genetic and target expression data indicate that CK2 promotes miRNA-mediated target silencing in a wide array of tissues and developmental stages.

CK2 is dispensable for the expression of miRNAs and miRISC factors

To explore where in the miRNA pathway CK2 functions, we first investigated whether CK2 regulates the biogenesis or stability of mature miRNAs. We performed deep sequencing of small RNAs in wild-type animals treated with vector, *kin-3* or *kin-10* RNAi and observe no significant differences in the global levels of mature miRNAs between empty vector and CK2 RNAi (Fig 2-3A, Fig 2-S4A and Table 2-S3). Furthermore, northern blot analysis of miR-48, miR-1, and *bantam* miRNA family ortholog, miR-58, in

wild-type and sensitized *alg-1*(*tm369*) animals confirmed that CK2 RNAi does not dramatically affect mature or precursor miRNA levels (Fig 2-3B, SI Appendix Fig 2-S4B,C). We also examined if CK2 regulates the accumulation or stability of core miRISC proteins. We detect no difference by western blot of endogenous ALG-1, AIN-1, VIG-1, TSN-1, and CGH-1 levels between empty vector and CK2 RNAi (Fig 2-3C). Together, these data indicate that CK2 functions downstream of miRNA biogenesis and is not required for stability of individual miRISC factors.

CK2 promotes miRISC association of mRNA targets

Defects in miRISC target silencing could result from compromised ability to bind mature miRNAs or mRNA targets. To determine if CK2 is required for miRNA or target binding to miRISC, we performed RNA-immunoprecipitation (RIP) using a strain expressing GFP::ALG-1 [56] and compared relative miRNA and target mRNA binding in CK2 RNAi versus empty vector and *gfp* RNAi controls (Fig 2-3D). We observe no significant differences in the levels of *let-7* or miR-48 associated with miRISC upon CK2 RNAi versus empty vector control RNAi (Fig 2-3E). Importantly, the target mRNAs of these miRNAs, *lin-41* and *daf-12*, show a ~2-fold reduction in miRISC association upon CK2 RNAi (Fig 2-3F). As expected, RNAi of *gfp*, which knocks down the expression of GFP::ALG-1, exhibited significant reduction of both miRNA and target mRNA levels (Fig 2-3F,G). Taken together, these data indicate that CK2 promotes efficient binding of target mRNAs, but not mature miRNAs, to miRISC.

CK2 phosphorylates miRISC cofactor CGH-1

Given our mass spectrometry data that CK2 physically associates with miRISC, we hypothesized that CK2 promotes miRISC target binding by phosphorylating one or more miRISC cofactors. We queried a *C. elegans* phospho-proteome dataset [57] and found peptides phosphorylated at CK2 motifs in CGH-1 and VIG-1. Since *cgh-1* loss of function mutants display developmental phenotypes that are easy to observe and CGH-1 has been implicated in promoting miRISC-target interactions [15], we first examined if CK2 physically interacts with CGH-1. Endogenous KIN-3 co-immunopurified with CGH-1::GFP in lysates from *C. elegans* fed *glp-1* RNAi to enrich the capture interactions in somatic tissues, the major sites of miRNA-mediated gene regulation (Fig 2-4A). *glp-1* encodes a *C. elegans* Notch family receptor [58] required for mitotic proliferation of germ cells and maintenance of germline stem cells [59]. *glp-1* RNAi severely disrupts early germline development yielding adult animals that are primarily composed of somatic cells. Mass spectrometry of CGH-1::GFP complexes confirmed the interaction of KIN-3 and additionally identified peptides corresponding to KIN-10 and the known CGH-1-interactor NHL-2 (SI Appendix Table S4).

To establish if CGH-1 could be phosphorylated by CK2, we performed in vitro kinase assays. CK2 phosphorylates full length CGH-1 in a manner dependent on CK2 activity, as inhibition of CK2 by addition of the CK2 inhibitor, TBB, or use of a catalytically dead KIN-3 (K67M) abrogates CGH-1 phosphorylation (Fig 2-4B). CGH-1 harbors four consensus motifs (S/TXXD/E) for CK2 phosphorylation (Fig 2-4C). In vitro kinase assays with short CGH-1 peptides containing each putative CK2 phosphorylation site indicate that site 1, containing serine 2 (S2), is the predominant site phosphorylated by CK2 (Fig 2-4D). Phospho-mass spectrometry of immunopurified CGH-1::GFP

recovered peptides containing phosphorylated S2 from wild-type, but not CK2 RNAi, animals, suggesting that site 1 is phosphorylated in vivo in a CK2-dependent manner (Table 2-S4). While S2 is not located in any functionally annotated domain of CGH-1, it is within an N-terminal intrinsically disordered region [60] that is conserved in DDX6 (Fig 2-S5). Intrinsically disordered regions have been hypothesized to be subject to post-translational modifications by kinases and phosphatases, which may regulate protein function [25,61]. In addition, serine 2 of CGH-1 is broadly conserved in orthologous proteins and conservation of phosphorylation at this site is supported by large-scale phospho-proteomic evidence in amphibians and mammals (Gnad et al., 2011; Gnad et al., 2007).

CK2 phosphorylation of CGH-1 promotes miRISC function

If CK2 phosphorylation of CGH-1 at S2 is important for CGH-1 to promote miRISC activity, then CGH-1 phospho-defective mutants should have compromised miRISC function. To test this hypothesis, we generated multi-copy CGH-1::GFP transgenic lines with phospho-defective (S2A) and phospho-mimic (S2D/E) mutations (hereafter referred to as S2 variants) (Fig 2-S6). Since *cgh-1* is critical in germline development and multi-copy transgenes are commonly silenced in the *C. elegans* germline, we examined the function of the S2 variants in the sensitized *let-7(mg279)* background using a temperature sensitive *cgh-1* point mutant, *cgh-1(tn691),* which phenocopies *cgh-1* loss-of-function at the non-permissive temperature of 25°C [62]. Alone, *cgh-1(tn691); let-7(mg279)* displays a partially penetrant protruding vulva phenotype (Pvul) that is significantly rescued by wild-type CGH-1::GFP expression but significantly exacerbated

by the phospho-defective S2A mutation (Fig 2-4E,F). Phospho-mimics yielded less conclusive data: S2D mutants have a phenotype similar to the non-transgenic control, while S2E mutants show partial, but significant, rescue. We also examined the effect of the S2 variants on the retarded alae phenotype of *cgh-1(tn691); let-7(mg279)* mutants. Expression of wild-type and S2E CGH-1::GFP significantly rescue alae defects, while the phospho-defective S2A mutants and the S2D mutant have defects similar to, or greater than, the non-transgenic control (Fig 2-4G). Thus, in two different tissues, the CGH-1 phospho-defective S2A mutation fails to complement miRNA mutant phenotypes. Based on the robust phenotype of the S2A mutants, we hypothesize that differential results with S2D and S2E may be attributed to incomplete phospho-mimicry by the substituted amino acids. Together, these data support the hypothesis that phosphorylation at S2 is important for miRNA-dependent CGH-1 function.

To determine the requirement for CK2 and phosphorylation of CGH-1 in a biological context where miRNA activity is modulated, we compared continuous development to post-dauer development. Continuous development from L1 to adulthood occurs in favorable environmental conditions. By contrast, adverse conditions promote entry into the stress-resistant and developmentally arrested dauer larva stage immediately after the L2 molt [63]. If dauer larvae encounter an environment suitable for growth, they resume normal development such that post-dauer cell divisions are identical to those occurring during continuous development [64,65]. Robustness to developmental interruption by dauer is due, in part, to enhanced activity of *lin-4* and *let-7* family miRNAs, such that *lin-4* can substitute for miR-48, miR-241, and miR-84 during post-dauer development but not continuous development [66]. Surprisingly,

enhancement of miRNA activity occurs without *alg-1*, as heterochronic phenotypes of *alg-1* mutants are efficiently suppressed post-dauer [66]. This suggests that modulatory proteins elevate the activity of the remaining ALG-2-containing miRISC. CK2 appears to contribute to this phenomenon, as *kin-3* RNAi significantly reduces post-dauer suppression of *alg-1(gk214)* alae defects (Fig 2-S7A). Interestingly, we observe that during post-dauer development, the retarded alae phenotype of S2A mutants is significantly suppressed, suggesting that CK2 modification of CGH-1 is dispensable during post-dauer development (Fig 2-S7B).

DISCUSSION

Here we report that subunits encoding the *C. elegans* casein kinase II, *kin-3* and *kin-10,* are required for effective activity of the miRNA pathway. Genetically, our analyses suggest that *kin-3* and *kin-10* promote the function of several miRNA/miRNA families that regulate diverse processes, including the conserved function of the *let-7* family of miRNAs in the control of animal development and cellular differentiation. Mechanistically, our analyses place CK2 function at the step of miRISC association with mRNA targets, downstream of miRNA biogenesis and loading into miRISC. Molecular assays indicate that CK2 subunits are required for target silencing and that their depletion compromises miRISC binding to *let-7* target mRNAs. Through the use of proteomic and in vitro methods we establish conserved miRISC cofactor, CGH-1, as a direct CK2 substrate at a conserved N-terminal serine residue (S2). Additional genetic analyses suggest that S2 is important for miRNA-dependent CGH-1 function in the *let-7* pathway. Since CGH-1 is part of a family of DEAD-box helicases that influence all

permutations of protein-protein, protein-RNA and RNA-RNA interactions, we propose that phosphorylation of CGH-1 by CK2 may influence miRISC target binding by (1) directly promoting miRISC interaction with targets, either by promoting CGH-1 association with miRISC, or by altering affinity of a CGH-1 associated-miRISC for targets, and/or (2) promoting CGH-1 association with factors involved with mRNA translation and decay. Biochemically, phosphorylation in the N-terminal disordered region of CGH-1 may affect these changes by triggering a conformational change that alters the intrinsic activity of CGH-1 or facilitates its association with other factors.

Intriguingly, our analysis of post-dauer development suggests that CK2 function in the *C. elegans* miRNA pathway is different than during continuous development. Post-dauer suppression of miRNA phenotypes requires CK2, but not CK2 phosphorylation of CGH-1 (Fig 2-S7B), suggesting that during post-dauer development, CK2 may have alternative miRNA pathway substrates. Alternatively, additional factors may dictate CK2 specificity during continuous development versus post-dauer development. Despite considerable efforts, the matter of whether CK2 is constitutively active or activated by specific signals remains controversial [67]. Therefore further investigation into CK2 function in the miRNA pathway during post-dauer development in may prove a useful system for extending our understanding of the regulation of CK2. As modulating miRNA activity is crucial for development and disease, dissecting the mechanisms that activate or antagonize CK2 activity in the miRNA pathway may also provide further insights into how the miRNA pathway is regulated.

MATERIALS AND METHODS

Detailed methods are available in Supplemental materials and methods.

***C. elegans* strains and plasmids.** All strains used in this study are listed in Table 2-S1. *C. elegans* were grown and maintained under standard laboratory conditions [68] and synchronized by hypochlorite treatment and overnight hatching in M9 buffer, except where indicated. Descriptions of plasmid construction and generation of transgenic animals are described in Supplemental Methods.

RNAi. All RNAi clones are from the Ahringer RNAi library [69], except the *kin-3* RNAi clone, which is from the *C. elegans* ORFeome RNAi library [70], and the *cgh-1* 3'UTR RNAi clone (pJK301), which was made for this study. Standard RNAi procedures [71] were followed. Specific modifications to RNAi conditions, including duration, strength, and temperature, are detailed in Supplemental Methods.

***mef-2* reporter intensity.** Images were captured on an Olympus BX61 epifluorescence compound microscope with a Hamamatsu ORCA ER camera using Slidebook 4.0.1 digital microscopy software (Intelligent Imaging Innovations) and processed in ImageJ with custom scripts.

Dauer induction and post-dauer phenotyping. Dauer Induction was achieved using crude dauer pheromone (Vowels and Thomas, 1994) for *kin-3(RNAi)* experiments and by starvation/crowding for experiments with *cgh-1(tn691); let-7(mg279)*. All strains were

maintained at 20°C. Strains in the *cgh-1(tn691); let-7(mg279)* background were shifted to 25°C at the L2 molt during continuous development or after selection of dauer larvae for post-dauer development . Additional phenotyping details are provided in Supplemental Methods.

CK2 in vitro kinase assays. Recombinant GST fusion proteins were generated and isolated from *E.coli* according to [72]. Purified recombinant proteins were analyzed by SDS-PAGE and Coomassie Blue to determine protein concentrations by comparison to BSA standards. For in vitro kinase reactions, 1 µg of recombinant CGH-1 was mixed with 500 nM recombinant KIN-3 (either WT or K67M mutant), 500 nM KIN-10, 4 µCi of P32 γATP, and 200 µM cold ATP in 20 mM Tris-HCl, pH7.5, 50 mM KCl, and 20 mM MgCl2. Reactions were incubated at room temperature for 30 min. To inhibit CK2 activity in vitro, TBB (4,5,6,7-tetrabromobenzotriazole) was added to 5 µM. To map the specific phosphorylation site on CGH-1: GST-tagged CGH-1 peptides comprised of the 20aa flanking the four putative CK2 phosphorylation sites were incubated with CK2 as described above (peptide sequences in Table 2-S2). All data were analyzed by SDS-PAGE and autoradiography.

Western blotting. Protein samples from synchronized populations were prepared by direct boiling in Novex Tris-Glycine SDS sample buffer (Invitrogen), supplemented with 0.1 M DTT, resolved on Novex Tris-Glycine gels (Invitrogen), and immobilized on Immobilon-FL transfer membrane (Millipore). Membranes were probed with custom rabbit polyclonal antibodies to ALG-1 (1:1000), AIN-1 (1:1000), CGH-1 (1:1000), GFP

(1:500), KIN-3 (1:500), TSN-1 (1:1000) and VIG-1 (1:1000), or γ-tubulin (Sigma LL-17) (1:2000). Peroxidase-AffiniPure goat anti-rabbit IgG secondary antibody was used at 1:10000 (Jackson ImmunoResearch Laboratories) for detection using Pierce ECL western Blotting Substrate (Thermo Scientific).

Antibody production. Custom polyclonal antibodies to *C. elegans* proteins were generated by immunizing rabbits with synthetic antigenic peptides conjugated to KLH (Proteintech). Antigenic peptide sequences are listed in Table 2-S2. Antibody to GFP was made using recombinant GFP purified from *E. coli*. The LIN-14 antibody was a gift from Gary Ruvkun [73].

Immunoprecipitation and mass spectrometry. Synchronized populations were frozen in liquid nitrogen and homogenized with a Mixer Mill MM 400 ball mill homogenizer (Retsch). Immunoprecipitations from homogenates were performed using guidelines described [74], with additional details provided in Supplemental Methods. Protein identification by mass spectrometry was performed as described [75].

RNA analyses. Samples were collected from synchronized populations at the following times: 48 hr post-L1 for mature miRNA, 44 hr post L1 for *lin-41* mRNA, 40 hr post L1 *daf-12* mRNA. Conditions for *kin-3*, *kin-10*, and *alg-1* RNAi are described in the RNAi methods. Total RNA isolation was conducted as described [76] For miRNA analysis: (1) Northern blotting was performed as described [77] using 1.5 µg total RNA and Starfire DNA probes (IDT) (Table 2-S2), (2) RT-qPCR analysis was conducted using miRNA

TaqMan assays and probes (hsa-let-7a cat#4427975, cel-miR-28 cat#4427975, U18 cat#4427975) (Applied Biosystems) as described [76] . For mRNA analysis cDNA was synthesized from total RNA using the Multiscribe Reverse Transcriptase Kit (Applied Biosystems). Specific mRNAs were analyzed by RT-qPCR using Power SYBR Green Master Mix 2X (Applied Biosystems). Relative miRNA and mRNA levels were calculated based on the ΔΔ2*Ct* method [78] using U18 and *eft-2* for normalization, respectively. RT-qPCR primer sequences and Starfire Probes are listed in Table 2-S2.

RNA-Immunoprecipitation (RIP). GFP-ALG-1 was immunoprecipitated from L4 stage (48 hr post L1) CT20 with 3E6 anti-GFP antibody (Invitrogen). For each RIP, antibody was cross-linked to Protein A Dynabeads (Invitrogen) and incubated at 4°C for 1 hr with homogenized sample. Beads were washed with RIP wash buffer (50 mM Tris-HCl pH 7.5, 200 mM KCl, 0.05% NP-40), then split for protein and RNA analyses. For protein analyses, SDS sample buffer (Novex Tris-Glycine, Invitrogen), was added directly to beads and incubated at 50°C for 10 min. Eluted proteins were supplemented with 0.1 M DTT, and incubated at 90°C for 5 min prior to western blotting. For RNA analysis, 1 ml of TRI-Reagent (Ambion) and 10 ng of Firefly Luciferase RNA (Promega) was directly added to beads and extracted as described in RNA Analyses, with extended RNA precipitation for 2 hr at -30°C. cDNA and miRNA taqmans were synthesized and analyzed as described in RNA Analyses. RT-qPCR analyses were normalized to firefly luciferase.

Small RNA sequencing and miRNA analysis. Samples were collected from synchronized populations of 48 hr post-arrested L1 fed L4440, *kin-3*, or *kin-10* RNAi. Total RNA was isolated as described in "RNA Analyses" section. Total RNA was submitted to the Beijing Genomics Institute for deep sequencing. Sequencing reads were processed to remove low quality reads and trim artificial adapter sequences following BGI protocols. Briefly, reads were marked and removed for low quality if they had (1) more than 4 bases whose quality score was lower than 10 or (2) more than 6 bases whose quality score was lower than 13. 3' adapter sequences were then trimmed from the remaining high-quality reads. Finally, reads were removed if they (1) lacked a 3' adapter sequence, (2) were 5'-3' adapter ligation products, (3) were 5'-5' adapter ligation products, (4) were shorter than 18 nt, or (5) contained only As (homopolymers). High-quality reads were aligned to the reference *C. elegans* genome version WS220 using Bowtie2 [79] with the following parameters: -f -N 0 -M 10. Reads that aligned with zero mismatches to one genomic locus were annotated to mature miRNA coordinates from miRBase Release 19 [4]. Mature miRNA read counts in each library were normalized to the number of mapped reads in the library; mature miRNA abundance is reported as reads per million mapped reads (RPM) (Table 2-S3). Raw sequence data are available through the NCBI's Gene Expression Omnibus using accession GSE66764.

SUPPLEMENTAL MATERIALS AND METHODS

Plasmids and Transgenic Strains. The *kin-3::gfp* reporter plasmid (pJK194*)* was generated by introducing the following fragments into pJK211 (a derivation of Fire

vector pPD49.26): *kin-3* endogenous promoter (2.0 kb fragment immediately upstream of the *kin-3* start codon), *kin-3* genomic coding region (2.2 kb fragment with mutated termination codon), *gfp* coding region (0.9 kb fragment with synthetic introns and termination codon), and *kin-3* endogenous 3′UTR (1.2 kb fragment immediately downstream of *kin-3* termination codon). The *Pmef-2::gfp::mef-2* 3'UTR reporter KP#1438 was generated by sub-cloning the following fragments into *gfp* 3'UTR reporter KP#1436 [55]: *mef-2* endogenous promoter (4.1kb fragment upstream of the *mef-2* coding region) and *mef-2* endogenous 3′UTR (2.2kb fragment downstream of *mef-2* termination codon). The two most 5' miR-1 sites in the *mef-2* 3'UTR were scrambled to abrogate seed recognition using SOE-PCR and subcloned into KP#1438 to create the *Pmef-2::gfp::mef-2 3'UTR (scrambled miR-1 sites I and II)* plasmid. The *cgh-1::gfp* reporter plasmid (pJK297) was generated by introducing the following fragments into pJK211: *cgh-1* endogenous promoter and genomic coding region (~3.7 kb fragment including 2.1 kb immediately upstream of the *cgh-1* start codon and the *cgh-1* coding region with mutated termination codon), *gfp* coding region (0.9 kb fragment with synthetic introns and termination codon), and *unc-54* 3'UTR (0.76 kb PCR fragment downstream of the *unc-54* termination codon which includes the 282 bp annotated *unc-54* 3'UTR. *cgh-1::gfp* S2 variants were generated from pJK297 by introducing site-specific mutations to alter serine to encode alanine (pJK789), aspartic acid (pJK787), or glutamic acid (pJK791) Reporter plasmids were used to generate multi-copy integrated transgenes as described [80].

The *cgh-1* 3'UTR RNAi clone (pJK301), which was made by subcloning a ~0.5 kb fragment amplified from the *cgh-1* 3'UTR into the vector, *L4440* [Primer I:

ACACTAGTTCTCATATCCCCAAACCTCCAAAACACACAGCGGCCGCCATATCCCCA AACCTCCAAAACAC (Not-I); Primer 2: AATGTGGTGCGGCTCAACAGAATAACTACAAATGCTAGCCGGCTCAACAGAATAAC TACA (Nhe I)] , followed by transformation into *E. coli HT115*.

Modified RNAi Conditions. Since several genes in this study are essential for development and/or fertility, feeding RNAi (1) duration (one versus two generations) and (2) strength (RNAi cultures were diluted with cultures from bacteria expressing the empty vector *L4440*) were optimized to achieve a balance of efficient knockdown and viability. For RNAi spanning two generations, two rounds of synchronization were performed. RNAi conditions for alae defect, seam cell number, adult collagen expression, lethargus, Rup, and Muv assays were as follows: *kin-3* RNAi (1 generation, no dilution), *kin-10* RNAi (2 generations, 1:1 dilution), *alg-1* RNAi (1 generation, 1:1 dilution), *ain-1* RNAi (2 generations, no dilution), and *nhl-2* RNAi (2 generations, no dilution). RNAi for *mef-2* GFP reporters were for two generations as follows, images of young adults were taken in the second generation: *kin-3* RNAi (P0 1:2 dilution, F1 1:1 dilution), *kin-10* RNAi (P0 and F1 1:1 dilution), *alg-1* RNAi (P0 vector, F1 1:1 dilution), and both *gfp* and *vector* RNAi (P0 and F1 no dilution). RNAi for LIN-14 western analysis was as follows: *kin-3* RNAi (1 generation, 1:2 dilution), *kin-10* RNAi (1 generation, 1:1 dilution), *alg-1* RNAi (2 generations: P0 at 1:2 and F1 at 1:1), and *lin-14* RNAi (1 generation, 1:1 dilution). Additional modifications to the standard feeding protocol include: (1) assays with QK005 were grown on vector RNAi for 36 hr, then transferred to undiluted RNAi for 120 hr prior to scoring ASEL neuron specification, (2) assays with

MT14119 were grown on vector RNAi until the L3 larval stage, then transferred to undiluted RNAi; adults were removed post-egg lay and their eggs scored for viability. Temperature modifications include: QK005, SD551, and QK039-QK044 were grown at 25°C. MT14119 was grown at 15°C.

Dauer Induction and Post-Dauer Phenotyping. For *kin-3* RNAi experiments *alg-1(gk214)* embryos isolated by alkaline-hypochlorite embryo extraction were plated directly on RNAi at 20˚C. For continuous development, animals were grown for 2-3 days to reach young adult stage. For post-dauer development post-dauer development, animals were grown on RNAi plates with crude dauer pheromone [81] in parallel to animals in continuous development. After 2-3 days, dauer were picked by morphology to fresh RNAi plates lacking pheromone. These post-dauer larvae were grown for an additional day to reach young adult stage. For comparison between continuous development and post-dauer development in QK039-QK044, strains were maintained at 20˚C. For continuous development, a synchronized population was obtained by an adult egg lay for 3-4 hrs at 20˚C. Adults were removed and progeny were kept at 20˚C for ~30 hrs (until the L2 molt). Larvae were then shifted to 25˚C where they grew for another 40-45 hrs (~24 hrs past L4 molt). For post-dauer development, dauers were selected from populations starved at 20˚C by treatment in 1% SDS for 20 minutes. Dauers were then grown on fresh plates at 25˚C for 48 hrs (~24 hrs past the L4 molt). For analysis of continuous development only QK039-QK044 were kept at 25˚C from adult egg lay. In all cases, each individual was categorized as having either no adult alae, gapped adult

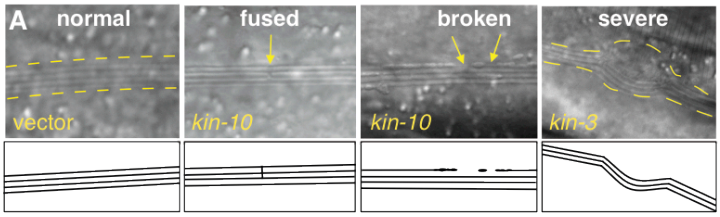
alae, or complete adult alae. One side was scored per individual using DIC optics on a Zeiss AxioImager D2 compound microscope.

Immunoprecipitation. The following adaptations of [74] were used to perform immunoprecipitations for mass spectrometry: *C. elegans* homogenates were suspended in lysis buffer (50 mM HEPES pH 7.4, 1 mM EGTA, 1 mM $MgCl_2$, 100 mM KCl, 10% glycerol, 0.05% NP-40 supplemented with Complete, Mini, EDTA-free Protease Inhibitor Cocktail tablet (Roche Applied Sciences), and clarified by centrifugation at 12,000X g for 12 min at 4°C. For immunoprecipitations, primary antibody conjugated to Dynabeads Protein A (Invitrogen) was incubated with homogenates at 4°C for 4 hr, then washed three times with wash buffer (50 mM HEPES pH 7.4, 1 mM EGTA, 1 mM $MgCl_2$, 300 mM KCl, 10% glycerol, 0.05% NP-40 supplemented with Complete, Mini, EDTA-free Protease Inhibitor Cocktail tablet (Roche Applied Sciences). Immunoprecipitated proteins were eluted from beads with three aliquots of 150 µL of 0.1 M glycine, pH 2.6. Eluates were neutralized with 150 µL of 2M Tris-HCl, pH 8.5, combined with 1/5 volume of 100% trichloroacetic acid, and precipitated overnight at 4°C. Proteins were pelleted by centrifugation at 20,000X g for 30 min and washed twice with acetone.

ACKNOWLEDGEMENTS

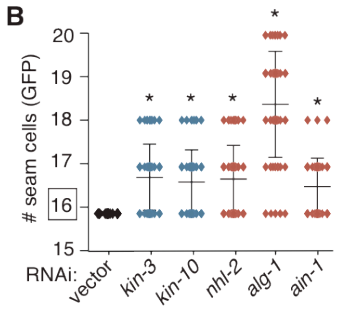
We thank Ken Inoki, Patrick Hu, and Alison Frand for discussions, Natasha Weiser for manuscript editing, and Dawen Cai for custom scripts used to analyze images in ImageJ. Indicated strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). We thank Gary Ruvkun for the LIN-14 antibody. This work was also supported by grants from the American Cancer Society (RSG RMC-125264) and NIH (GM088565) to JKK, National Center for Research Resources (5P41RR011823) and NIH (8 P41 GM103533) to JRY, and an Early Career Grant (C62241) from Central Michigan University to XK.

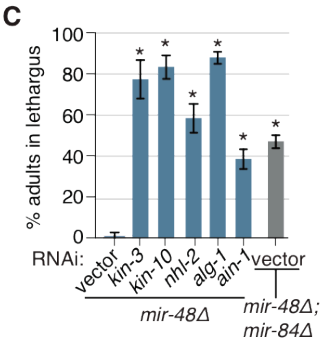
Figure 2-1. CK2 genetically interacts with the miRNA pathway. (A-B) CK2 depletion results in phenotypes associated with reduced miRNA pathway function: (A) RNAi of *kin-3*, *kin-10*, or *alg-1* significantly increases defective alae versus empty vector control (two-tailed Fisher's exact test $p<0.0001$). (B) RNAi of *kin-3*, *kin-10*, *nhl-2*, *alg-1*, and *ain-1* in animals expressing a seam cell gfp reporter *(Pscm::gfp)* all exhibit significant seam cell hyperplasia post L4 versus empty vector control (asterisk: two-tailed Student's *t*-test $p<0.001$, mean and standard deviation (SD) plotted, n=50). (C-J) CK2 depletion enhances miRNA mutant defects: (C) RNAi of *kin-3*, *kin-10*, *nhl-2*, *alg-1*, and *ain-1* all exhibit significant enhancement of adult animals in lethargus versus empty vector control (asterisk: two-tailed Student's *t*-test of biological replicates $p<0.05$, mean and SD plotted, n≥50 per replicate). Adult animals inappropriately entering lethargus were scored every 2h from 60 hr to 72 hr post L1. (D) *kin-3* and *kin-10* RNAi enhance Rup of *let-7*, *let-7* family, and miRISC factor mutants at 72 hr post L1, 20°C. Heatmap represents mean percent Rup of biological replicates (n≥50 per replicate). (E) Rup in homozygous *kin-10* deletion mutants is significantly greater than *hT2[qIs48]* balanced *kin-10/+* siblings (two-tailed Fisher's exact test $p\leq0.003$). (F-G) *kin-3* and *kin-10* RNAi Rup enhancement in *let-7* and *mir-48* mutants is dependent on their targets: (F) Rup enhancement of *kin-3 and kin-10* RNAi in *let-7(mg279)* is decreased in the *lin-41(ma104)* background (mean and SD of biological replicates plotted, n≥37 per replicate). (G) Rup enhancement of *kin-3* and *kin-10* RNAi in *mir-48(n4097*) is decreased in the *hbl-1(mg285)* background (mean and SD of biological replicates, n≥56 per replicate). (H) RNAi of *kin-3*, *kin-10*, *nhl-2*, and *alg-1* all significantly enhance Muv versus empty vector control (asterisk: two-tailed Student's *t*-test of biological replicates $p<0.05$, mean and SD plotted, n≥94 per replicate). (I) *kin-3* and *kin-10* RNAi increase the penetrance of ASEL misspecification in neuronal RNAi-competent *lsy-6*(*ot150*) strain indicated by the lack of P*lim-6*::*gfp* expression in ASEL (Fisher's exact test $p<0.0001$ for *kin-3* and *alg-1* RNAi, $p=0.0002$ for *kin-10* RNAi) (J) *kin-3* and *kin-10* RNAi enhance embryonic lethality in *mir-35-41*(*nDf50*) (asterisk: two-tailed Fisher's exact test $p<0.001$, n≥145).

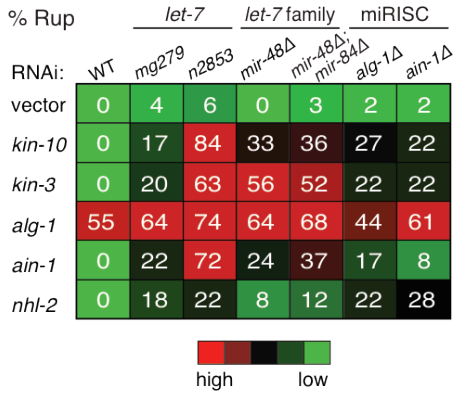


% alae defect at L4 molt

RNAi	*n*	Normal	Defect	Fused	Broken	Severe
vector	35	100	0	0	0	0
kin-3	39	38	62	18	28	16
kin-10	36	31	69	17	30	22
alg-1	36	14	86	11	47	28

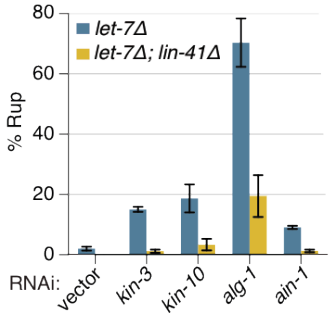


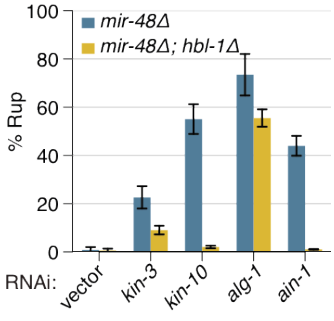


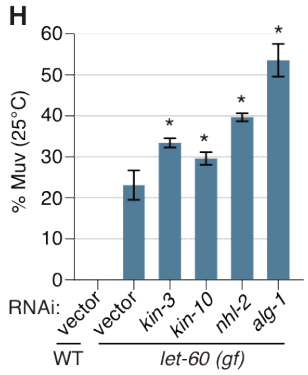


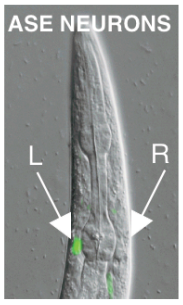
E

Genotype	*n*	# Rup	% Rup
kin-10 (ok1751)/+; let-7 (mg279)	36	0	0.0%
kin-10 (ok1751); let-7 (mg279)	13	4	30.8%
kin-10 (ok2031)/+; let-7 (mg279)	58	1	1.7%
kin-10 (ok2031); let-7 (mg279)	47	11	23.4%









% ASEL neurons expressing *Plim-6::gfp* in *lsy-6Δ*

RNAi:	ON (L R)	OFF (L R)	*n*
vector	87	13	284
kin-3	63	37	147
kin-10	66	34	62
alg-1	63	37	130

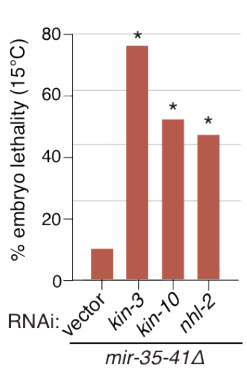


Figure 2-2. CK2 is required for miRNA target silencing. (A) Abridged table of miRNAs and their target mRNAs examined in this study. (B) *kin-3 and kin-10* RNAi attenuate silencing of *let-7* family targets *lin-41* and *daf-12*: *lin-41* mRNA levels are significantly elevated in *kin-3 and kin-10* RNAi versus vector RNAi (44 hr post L1, 20°C) (left panel); *daf-12* mRNA levels are substantially elevated in *kin-3 and kin-10* RNAi versus vector RNAi (40 hr post L1, 20°C) (right panel). (asterisk: one-tailed Student's *t*-test of biological replicates $p<0.050$, mean and SD plotted). (C) *kin-3 and kin-10* RNAi attenuates silencing of *lin-4* target *lin-14*. LIN-14 is up-regulated in *kin-3* and *kin-10* RNAi versus empty vector control in L2 (16 hr to 20 hr post L1). γ-tubulin used as a loading control. (D-E) *kin-3 and kin-10* RNAi attenuate silencing of miR-1 target *mef-2* in a miR-1-dependent manner. (D) Reporter constructs shown with wild-type and scrambled miR-1 sites (left). Arrows indicate vulva in representative images (right). (E) *mef-2* reporter quantification. RNAi of *kin-3, kin-10 and alg-1* all significantly increase GFP signal of the wild-type reporter versus vector ($p<0.05$, mean and SD plotted, $n>10$), but not the scrambled reporter ($p\geq0.60$).

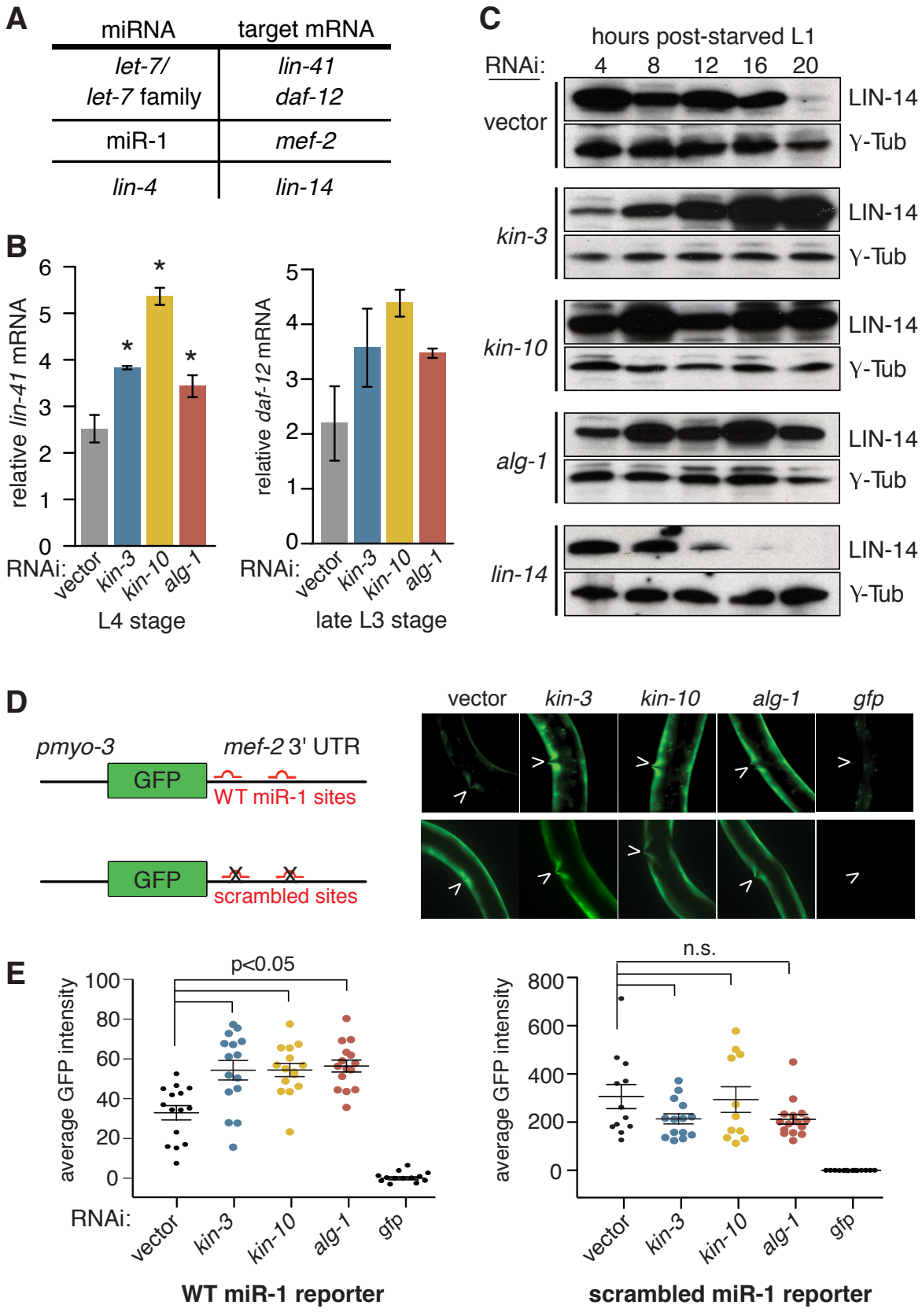

A
miRNA
target mRNA
let-7/ let-7 family
lin-41 daf-12
miR-1
mef-2
lin-4
lin-14
B
relative lin-41 mRNA
relative daf-12 mRNA
RNAi:
vector
kin-3
kin-10
alg-1
L4 stage
late L3 stage
C
hours post-starved L1
RNAi: 4 8 12 16 20
vector
kin-3
kin-10
alg-1
lin-14
LIN-14
γ-Tub
D
pmyo-3
mef-2 3' UTR
GFP
WT miR-1 sites
scrambled sites
vector
kin-3
kin-10
alg-1
gfp
E
p<0.05
n.s.
average GFP intensity
WT miR-1 reporter
scrambled miR-1 reporter

Figure 2-3. CK2 promotes miRISC target binding. (A-B) CK2 does not affect miRNA levels: (A) *kin-3* RNAi does not affect global mature miRNA abundances as quantified by deep sequencing (reads per million mapped reads: RPM). (B) Global analysis in (A) is supported by northern blotting: *kin-3* and *kin-10* RNAi do not substantially alter levels of precursor or mature miR-48 in wild-type or *alg-1*(*tm369*) animals. (C) CK2 RNAi does not considerably affect miRISC factor levels. Western analysis of core miRISC proteins in *kin-3* and *kin-10* RNAi are similar to empty vector control. (D-F) CK2 affects miRISC binding to target mRNAs: (D) Schematic and western analysis of GFP::ALG-1 RNA immunoprecipitation (RIP) from L4 (48 hr post L1, 20°C) lysates of empty vector versus *kin-3* and *kin-10* RNAi; gfp RNAi controls for RIP; γ-tubulin controls for protein input. (E) Levels of *let-7* and miR-48 associated with GFP::ALG-1 are not significantly different in *kin-3* and *kin-10* RNAi versus empty vector. (F) Levels of *lin-41* and *daf-12* mRNA associated with GFP::ALG-1 are decreased in *kin-3* and *kin-10* RNAi versus empty vector. RIP RNAs were normalized to spiked-in firefly luciferase mRNA (asterisk: one-tailed Student's *t*-test of biological replicates $p<0.05$, mean and SD plotted).

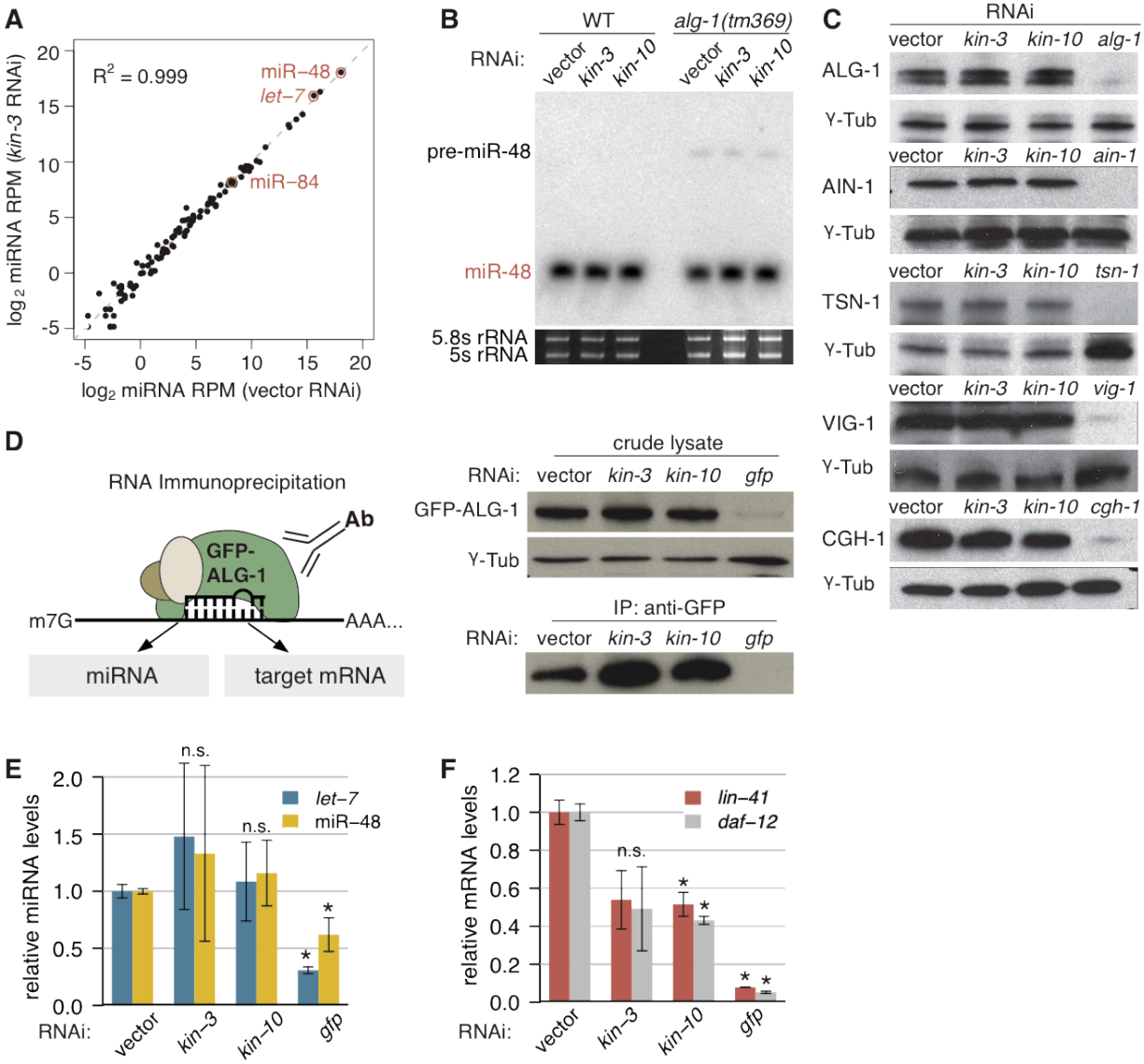

A
log2 miRNA RPM (kin-3 RNAi)
log2 miRNA RPM (vector RNAi)
R2 = 0.999
miR–48
let–7
miR–84
B
WT
alg-1(tm369)
RNAi:
vector
kin-3
kin-10
pre-miR-48
miR-48
5.8s rRNA
5s rRNA
crude lysate
GFP-ALG-1
Y-Tub
IP: anti-GFP
C
RNAi
vector
kin-3
kin-10
alg-1
ALG-1
ain-1
AIN-1
tsn-1
TSN-1
vig-1
VIG-1
cgh-1
CGH-1
D
RNA Immunoprecipitation
Ab
GFP-ALG-1
m7G
AAA...
miRNA
target mRNA
E
relative miRNA levels
let–7
miR–48
n.s.
F
relative mRNA levels
lin–41
daf–12

Figure 2-4. CK2 phosphorylates miRISC factor CGH-1 at serine 2. (A) KIN-3 associates with CGH-1 in the soma. CK2 catalytic subunit, KIN-3, co-immunopurifies with CGH-1::GFP in animals fed *glp-1* RNAi to prevent germline proliferation. (B) CGH-1 is phosphorylated by CK2 in vitro. Autoradiogram of GST-purified wild-type CGH-1 incubated with GST-purified CK2 comprised of wild-type or ATP-binding site mutant KIN-3 (K67M). TBB: CK2 inhibitor (4,5,6,7-tetrabromobenzotriazole). (C) CGH-1 harbors four CK2 recognition motifs (sites 1-4). (D) Site 1 (serine 2) is phosphorylated by CK2 in vitro. GST-tagged CGH-1 peptides comprised of 20 residues flanking each putative CK2 phosphorylation site were incubated in vitro with CK2 and analyzed by SDS-PAGE and autoradiography. ^{32}P: autoradiogram. CB: Coomassie Blue stained gel. (E-F) Genetic analysis of CGH-1 serine 2 (S2) phospho-variants in *cgh-1(tn691)ts; let-7(mg279)* suggests phosphorylation of CGH-1 at S2 promotes miRISC function (E) Representative images of Protruding vulva (Pvul). Adult vulva indicated by arrow (120 hr post-L1, 25°C). (F) Quantitation of Pvul. Compared to *cgh-1(tn691)ts;let-7(mg279)* with no transgene the *cgh-1:gfp* (S2) transgene significantly rescues Pvul. S2A mutants (Lines 1 and 2) significantly enhance Pvul (asterisk: two-tailed Fisher's exact test $p<0.0001$ for S2 variants versus "No TG", mean and SD plotted for three technical replicates within a single experiment, n≥95). (G) S2 and S2E significantly rescue alae defects of *cgh-1(tn691)ts;let-7(mg279)*. S2A mutants significantly enhance (Line 1) or have no significant effect (Line 2) on defects (asterisk: two-tailed Fisher's exact test $p<0.0001$ for S2 variants versus "No TG"). TG: transgene; S2A Lines 1 and 2: phospho-defective Ser-to-Ala mutation; S2D/E: phospho-mimic Ser-to-Asp/Glu mutations.

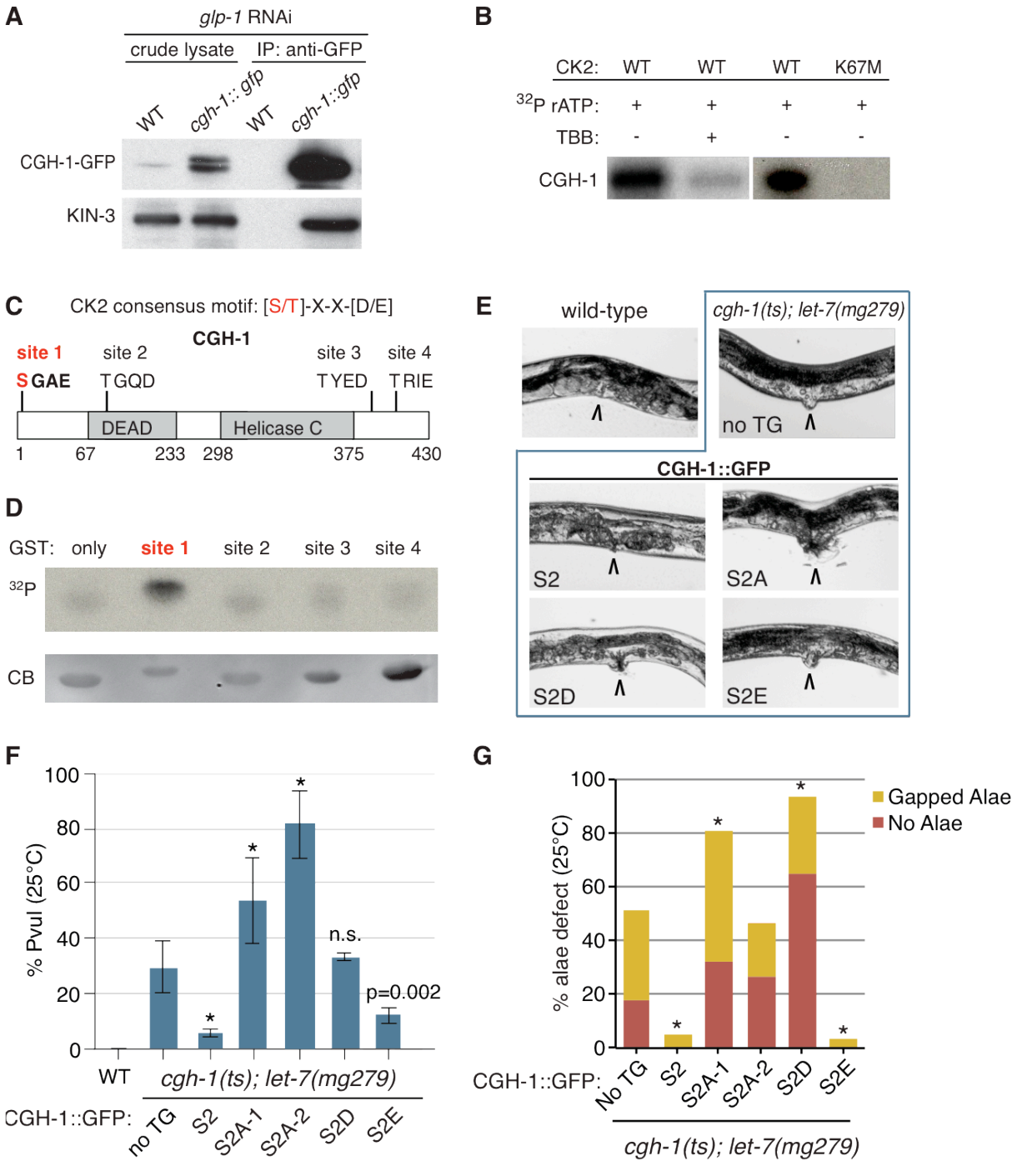

A
glp-1 RNAi
crude lysate
IP: anti-GFP
WT
cgh-1::gfp
WT
cgh-1::gfp
CGH-1-GFP
KIN-3
B
CK2: WT WT WT K67M
32P rATP: + + + +
TBB: - + - -
CGH-1
C
CK2 consensus motif: [S/T]-X-X-[D/E]
CGH-1
site 1
site 2
site 3
site 4
SGAE
TGQD
TYED
TRIE
DEAD
Helicase C
1
67
233
298
375
430
D
GST: only
site 1
site 2
site 3
site 4
32P
CB
E
wild-type
cgh-1(ts); let-7(mg279)
no TG
CGH-1::GFP
S2
S2A
S2D
S2E
F
% Pvul (25°C)
100
80
60
40
20
0
*
*
*
n.s.
p=0.002
WT
cgh-1(ts); let-7(mg279)
CGH-1::GFP:
no TG
S2
S2A-1
S2A-2
S2D
S2E
G
% alae defect (25°C)
100
80
60
40
20
0
*
*
*
Gapped Alae
No Alae
CGH-1::GFP:
No TG
S2
S2A-1
S2A-2
S2D
S2E
cgh-1(ts); let-7(mg279)

Figure 2-S1. RNAi efficiently knocks down CK2. (A) *kin-10* mRNA is efficiently depleted by *kin-10* RNAi at L3 (F1 RNAi, 1:1 dilution with vector) and young adult (P_0 RNAi, full strength). (B) KIN-3 protein is efficiently depleted in late L4-young adult by *kin-3* RNAi (P0 RNAi, 1:1 dilution with vector).

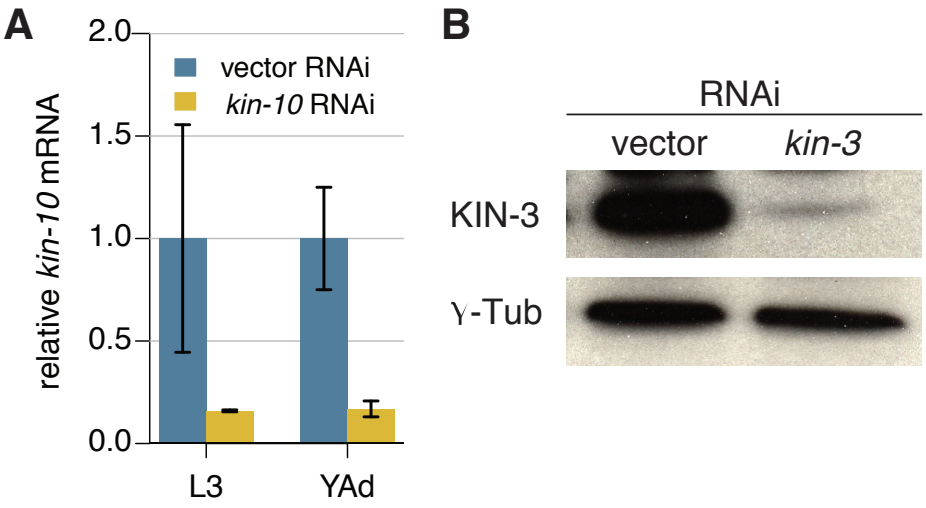
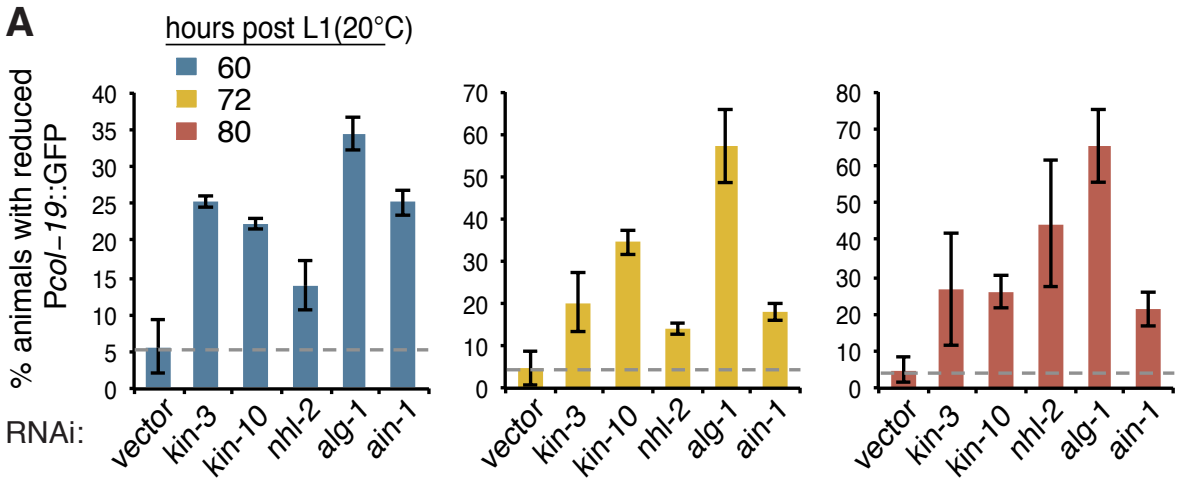


Figure 2-S2. *kin-3* and *kin-10* RNAi cause retarded defects in miRNA-mediated adult hypodermal remodeling. (A) *kin-3* and *kin-10* RNAi delay expression of adult-specific P*col-19*::*gfp* reporter. GFP expression was substantially decreased in populations fed *kin-3* and *kin-10* RNAi versus vector at time points spanning young adult through second day gravid (60 hr, 72 hr and 84 hr post L1 at 20°C). Mean and standard deviation of biological replicates are plotted (n>50 each). (B) KIN-3 is broadly expressed in larval animals. Top panels: Fluorescence microscopy of KIN-3::GFP driven by a 2 kb *kin-3* endogenous promoter. Bottom panels: DIC.



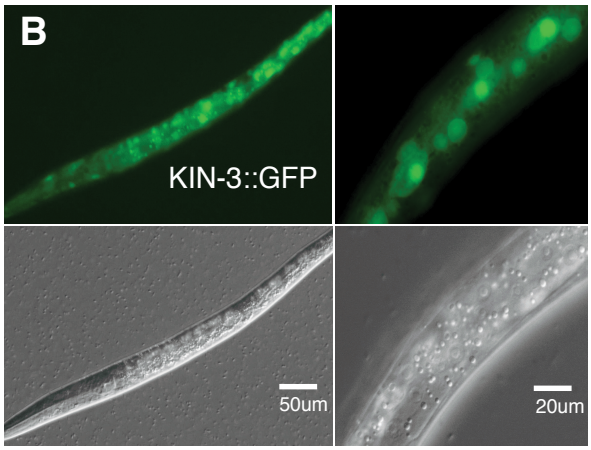


Figure 2-S3. RNAi treatments do not cause substantial developmental delay by L2 stage. Representative images of larvae used in LIN-14 western Analysis taken at the same magnification. Substantial growth observed between 8 hr and 20 hr suggests a lack of RNAi-induced developmental delay.

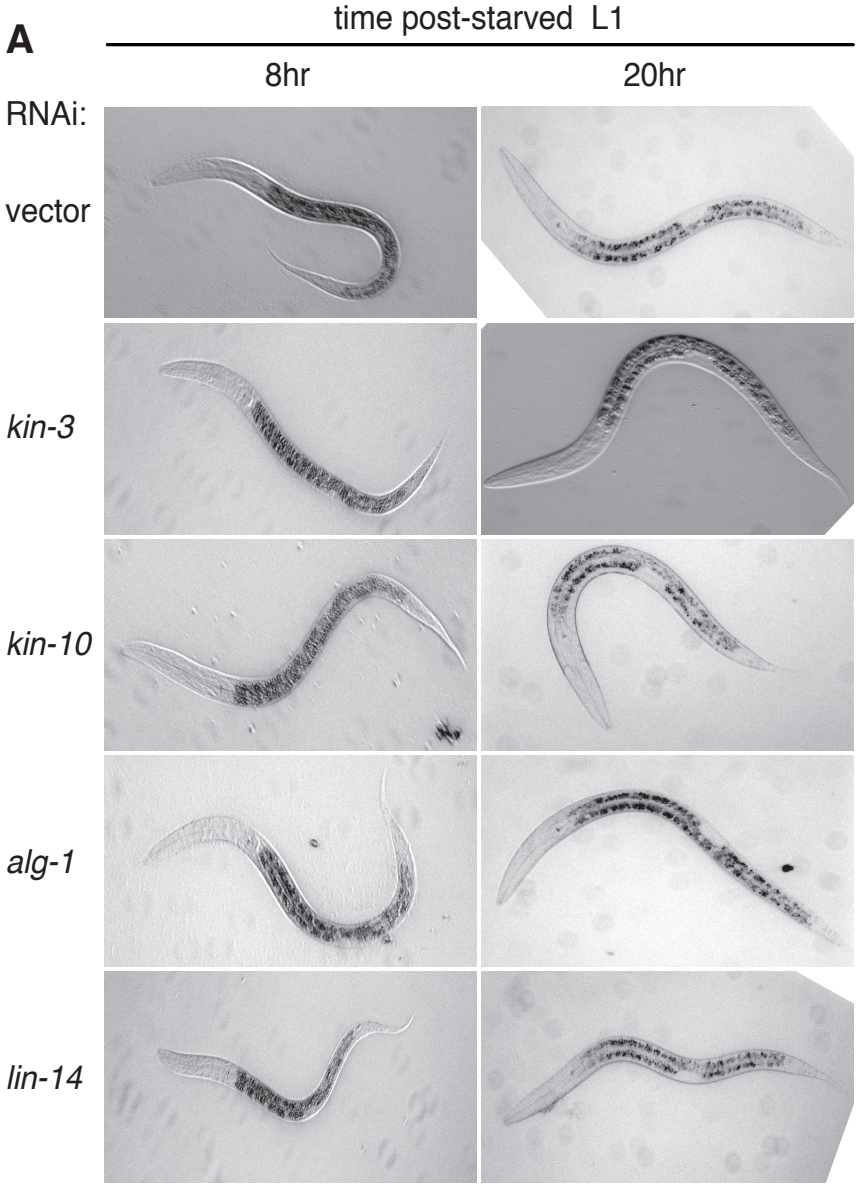


Figure 2-S4. CK2 does not affect miRNA levels. (A) *kin-10* RNAi does not affect global miRNA abundance. Mature miRNA levels are quantified by deep sequencing and represented as number of reads mapping to each mature miRNA normalized to total mapped library size in millions (RPM). (B) Global analysis in (A) was confirmed by northern blotting: *kin-3* and *kin-10* RNAi do not alter levels of precursor or mature miR-1 or miR-58 in wild-type or *alg-1*(*tm369*).

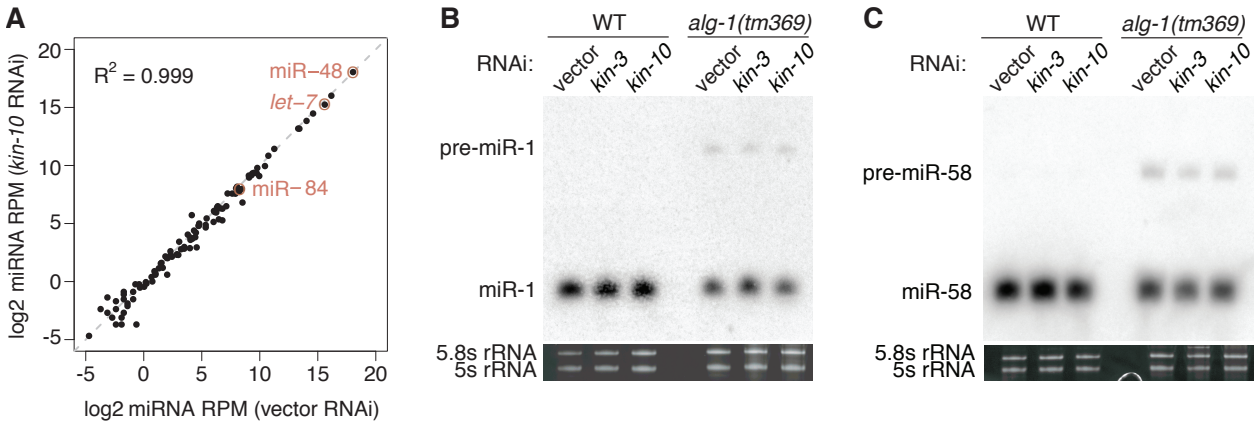


Figure 2-S5. CGH-1 and DDX6 have N-terminal intrinsically disordered regions that contain S2. Amino acid sequence of (A) CGH-1 and (B) DDX are plotted on the x-axis. Regions of the protein above the dotted line, highlighted in orange, are predicted to be intrinsically disordered. S2 is marked with a blue asterisk.

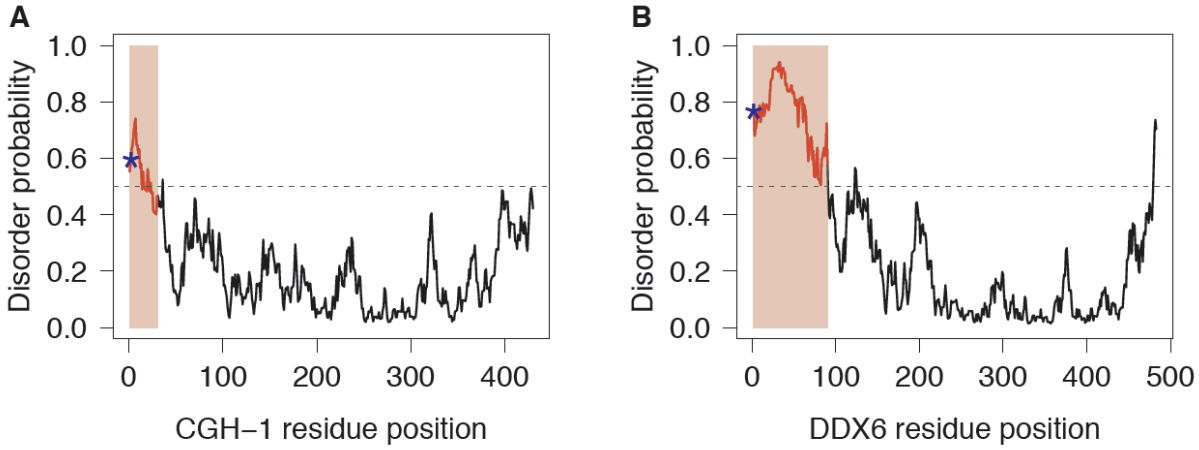


Figure 2-S6. Relative CGH-1::GFP expression of S2 variants. CGH-1::GFP expression was determined by SDS-PAGE and western blotting lysates of young adults (52 hr 20°C) using antibodies against GFP and endogenous CGH-1. γ-Tubulin loading control. (A) Soma-enriched expression of wild-type and S2 variant CGH-1::GFP expressing strains. *glp-1* RNAi was used to deplete germline. Two exposures of the anti-CGH-1 western blot are shown to best illustrate expression of either CGH-1::GFP (exposure 1) or endogenous CGH-1 (exposure 2). (B) Expression of CGH-1::GFP. Band specificity demonstrated in S2 fed *cgh-1* RNAi [Lane 1] or *gfp* RNAi [Lane 2], to knockdown endogenous *cgh-1* or both the *cgh-1::gfp* transgene and endogenous *cgh-1*, respectively, compared to no RNAi treatment [Lane 3]. Wild-type CGH-1::GFP = S2. Phospho-defective CGH-1::GFP= S2A Lines 1 and 2. Phospho-mimic CGH-1::GFP= S2D and S2E.

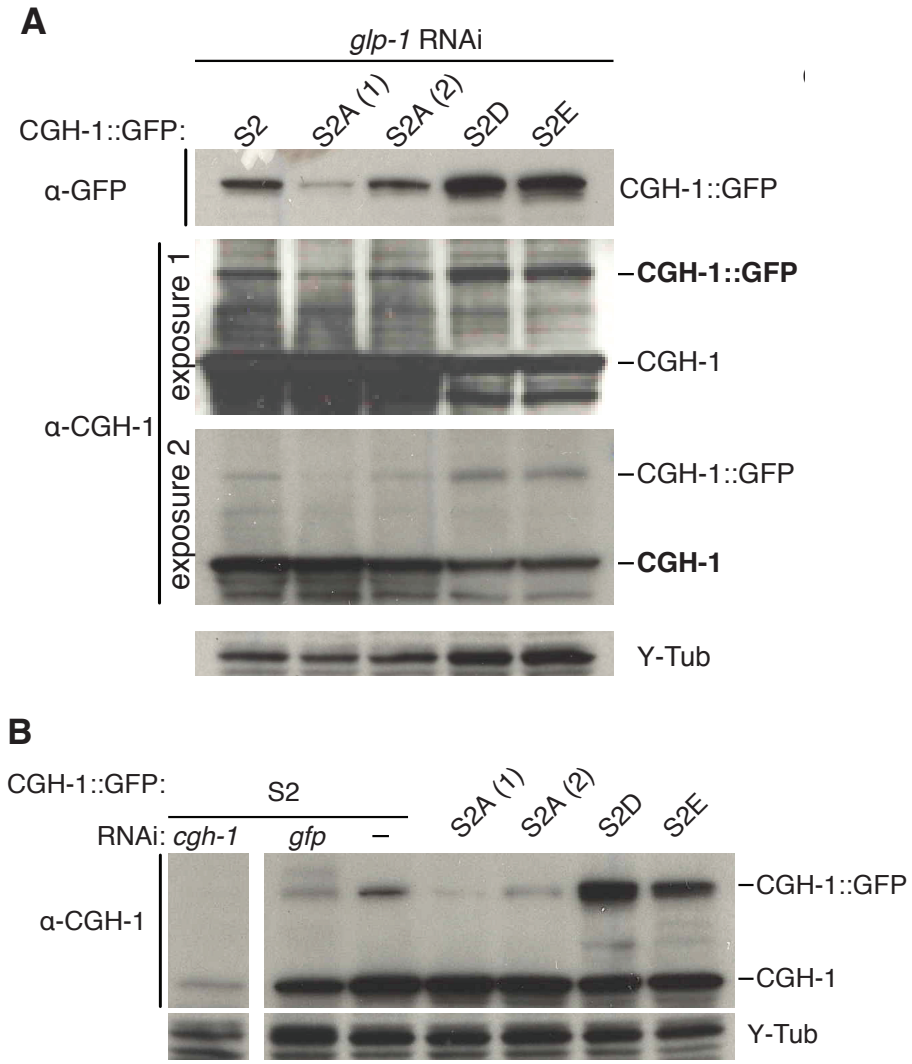


Figure 2-S7. **Post-dauer suppression of miRNA phenotypes requires KIN-3, but not CK2 phosphorylation of CGH-1. (**A) KIN-3 is required for post-dauer suppression of *alg-1(gk214)* alae defects. *alg-1(gk214)* on vector RNAi significantly suppress alae defects in post-dauer development (PDD). *kin-3* RNAi abrogates suppression of defective alae phenotypes in PDD (significance determined by two-tailed Student's *t*-test of biological replicates, mean and standard deviation are plotted, n≥39 per replicate). (B) Phosphorylation of CGH-1 is required only during continuous development (CD), and not during post-dauer development (PDD). Strains were grown at the permissive temperature until the L2 molt (for CD) or dauer (for PDD) (see Methods). Asterisk indicates p<0.0001 by two-tailed Fisher's exact test, n>60, each experiment was carried out at least twice. For all: TG= transgene, S2A Lines 1 and 2 = phospho-defective, S2D/E = phospho-mimic.

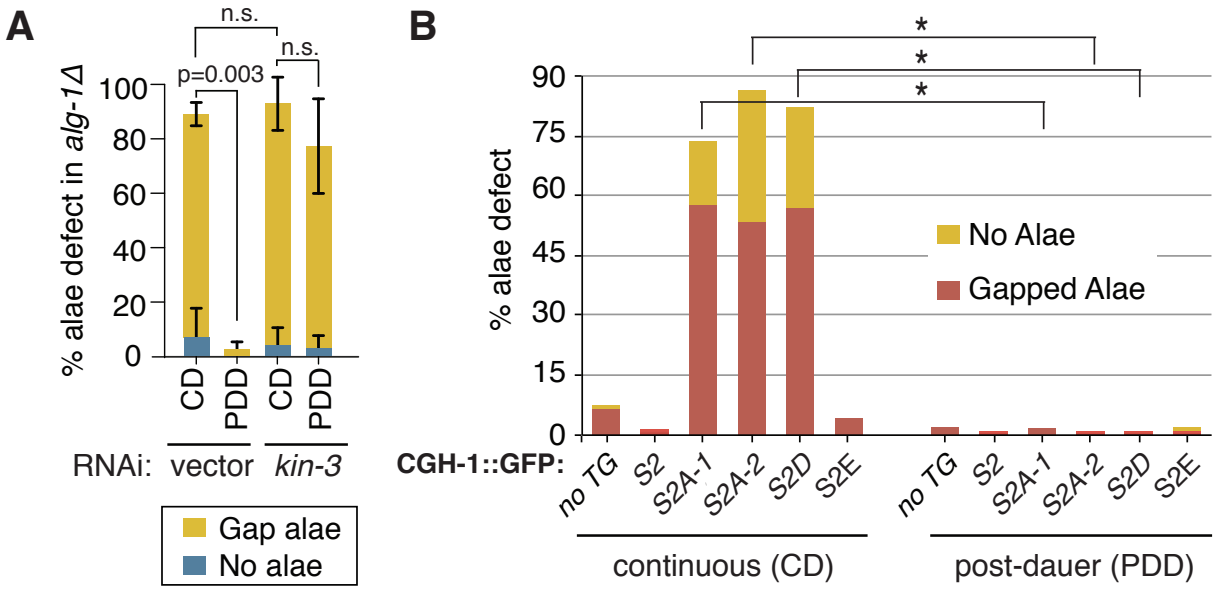


Table 2-S1. *C. elegans* strains used in this study.

Table S1

Strain ID	Genotype	Source
N2	*C. elegans* wild isolate; wild-type reference strain (WT)	CGC*
MH2385	*ain-1(ku322) X*	CGC
QK051	*ain-1(tm3681) X*	NBRP**
GS5217	*alg-1(gk214) X*	IS Greenwald
QK045	*alg-1(tm369) X*	NBRP
QK039	*cgh-1(tn691) III; let-7(mg279) X*	this study
QK040	*cgh-1(tn691) III; let-7(mg279) X; xkIs26 [Pcgh-1::cgh-1(WT)::GFP::unc-54 3'UTR]*	this study
QK041	*cgh-1(tn691) III; let-7(mg279) X; xkIs27 [Pcgh-1::cgh-1(S2A)::GFP::unc-54 3'UTR]*	this study
QK042	*cgh-1(tn691) III; let-7(mg279) X; xkIs28 [Pcgh-1::cgh-1(S2A)::GFP::unc-54 3'UTR]*	this study
QK043	*cgh-1(tn691) III; let-7(mg279) X; xkIs29 [Pcgh-1::cgh-1(S2D)::GFP::unc-54 3'UTR]*	this study
QK044	*cgh-1(tn691) III; let-7(mg279) X; xkIs30 [Pcgh-1::cgh-1(S2E)::GFP::unc-54 3'UTR]*	this study
QK032	*eri-1(mg366) IV; mir-48(n4097) V*	this study
CT11	*hbl-1(mg285) X*	CGC
RG365	*him-1(e879) I; veIs13[col-19::gfp; rol-6(su1006)] V*	AE Rougvie
VC1280	*kin-10(ok1751) I/hT2 [bli-4(e937) let-?(q782) qIs48](I;III)*	CGC
VC1609	*kin-10(ok2031) I/hT2 [bli-4(e937) let-?(q782) qIs48](I;III)*	CGC
GR1432	*let-7(mg279) X*	CGC
MT7626	*let-7(n2853) X*	CGC
SD551	*let-60(ga89) IV*	CGC
CT8	*lin-41(ma104) I*	CGC
QK004	*lin-41(ma104) I; let-7(mg279) X*	this study
MT14119	*mir-35-41(nDf50) II*	CGC
MT13650	*mir-48(n4097) V*	CGC
QK003	*mir-48(n4097) V; hbl-1(mg285) X*	this study
MT13652	*mir-48 (n4097) V; mir-84(n4037) X*	CGC
MT13651	*mir-84(n4037) X*	CGC
OH3646	*otIs114 [Plim-6::gfp] I; lsy-6(ot150) V*	CGC
QK005	*otIs114 [Plim-6::gfp] I; lsy-6(ot150) V; nre-1(hd20) lin-15b(hd126) X*	this study
VH624	*rhIs13 [unc-119::gfp, dpy-20(+)] V; nre-1(hd20) lin-15b(hd126) X*	CGC
JR672	*wIs54[scm::gfp] V*	JH Rothman
QK006	*xkIs25 [Pkin-3::kin-3::GFP::kin-3 3'UTR]*	this study
QK052	*xkIs31 [Pmyo3::gfp::mef-2 3'UTR]*	this study
QK053	*xkEx9 [Pmyo-3::gfp::mef-2 3UTR (scrambled miR-1 sites I and II)]*	this study
CT20	*zaIs5 [alg-1P::GFP::alg-1 + pRF4(rol-6(su1006))]*	CGC

*CGC - Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN.
**NBRP - National Bioresource Project for *C. elegans*, Tokyo Women's Medical University School of Medicine, Japan.

Table 2-S2. Oligos, Probes and Peptides used in this study.

Table S2

DNA Oligos	
ACGCTCGTGATGAGTTCAAG	*eft-2* qPCR forward
ATTTGGTCCAGTTCCGTCTG	*eft-2* qPCR reverse
GGTTCCAAATGCCACAAGAG	*lin-41* qPCR forward
AGGTCCAACTGCCAAATCAG	*lin-41* qPCR reverse
GATCCTCCGATGAACGAAAA	*daf-12* qPCR forward
CTCTTCGGCTTCACCAGAAC	*daf-12* qPCR reverse
CTCACTGAGACTACATCAGC	firefly luciferase qPCR forward
TCCAGATCCACAACCTTCGC	firefly luciferase qPCR reverse
TCAATTCGTTCCAAAACTCTAcG	*kin-10* qPCR forward
GTATTTCCGCCGCTGTTTCC	*kin-10* qPCR reverse
CTTCCAGGGAATTCTCGACCG	*cgh-1* qPCR forward
GCATGAACGAAGTGACGGTG	*cgh-1* qPCR reverse

Starfire Probes	
TACATACTTCTTTACATTCCA	miR-1
AACTATACAACCTACTACCTCA	*let-7*
TCGCATCTACTGAGCCTACCTCA	miR-48
TGCCGTACTGAACGATCTCA	miR-58

Antigenic Peptides	
QAGSLAPGVPIGNTSVSI(C)	ALG-1
WGDPPLSDVQYPLQPHASF(C)	AIN-1
(C)IEPIPKTVDPKLYVADQQLVDA	CGH-1
MPPIPSRARVYAEVNPSRP(C)	KIN-3
(C)AEGLALADHRREPRLQTLVNDY	TSN-1
(C)GRNNTPFNASDDAFPALGAK	VIG-1

CGH-1 GST Peptides	
MSGAEQQQIVPANNGDENWK	site 1
QEASIGVALTGQDILARAKN	site 2
LGVAINLITYEDRHTLRRIE	site 3
RRIEQELRTRIEPIPKTVDP	site 4

Table 2-S3. Quantification of mature miRNAs.

Table S3

	Normalized reads per million mapped reads		
miRNA	**vector**	***kin-3* RNAi**	***kin-10* RNAi**
let-7	49,795.02	63,690.70	38,749.08
lin-58	268,688.59	271,742.44	270,386.06
lsy-6	0.61	0.24	0.85
mir-1	73,658.84	81,012.21	65,989.66
mir-1018	2.47	1.15	2.20
mir-1019	0.11	0.10	0.39
mir-1020	1.59	1.60	1.31
mir-1022	659.16	595.35	637.46
mir-1817	0.15	0.04	0.00
mir-1819	40.48	33.35	25.28
mir-1820	2.01	1.15	1.62
mir-1821	0.76	0.45	0.85
mir-1822	0.19	0.52	0.31
mir-1823	0.19	0.07	0.08
mir-1824	1.14	1.56	0.97
mir-1828	0.00	0.00	0.00
mir-1829.2	110.01	69.58	77.61
mir-1830	0.80	1.87	0.70
mir-1834	1.18	1.01	0.89
mir-2207	0.15	0.10	0.00
mir-2208.2	0.53	0.52	0.35
mir-2209.1	7.28	5.52	5.10
mir-2209.2	0.00	0.04	0.00
mir-2209.3	0.00	0.04	0.04
mir-2210	0.30	0.45	0.54
mir-2211	1.02	0.97	0.73
mir-2212	4.17	4.51	4.06
mir-2213	0.30	0.17	0.16
mir-2216	0.53	0.45	0.54
mir-2219	0.38	0.24	0.27
mir-2220	0.11	0.07	0.16
mir-228	10,911.28	8,888.95	9,202.60
mir-229	76.02	79.40	61.76
mir-230	883.70	573.97	882.77
mir-231	26.48	26.13	27.79
mir-232	4.93	6.94	6.11
mir-235	364.48	282.23	112.09
mir-237	94.42	103.31	79.43
mir-238	138.54	127.08	89.90

mir-239.1	69.04	77.39	63.66
mir-239.2	109.52	63.44	38.69
mir-240	23.60	24.50	7.69
mir-241	10,173.44	10,289.80	9,281.79
mir-242	3.98	3.75	4.10
mir-243	8.31	10.27	10.78
mir-244	11.27	16.24	7.89
mir-245	3.11	2.46	2.28
mir-246	11.19	12.67	7.11
mir-247	1.90	0.97	1.86
mir-248	66.31	70.90	67.72
mir-249	0.30	0.28	0.46
mir-251	15.74	10.03	7.34
mir-252	299.08	298.78	211.61
mir-259	2.01	1.98	1.51
mir-260	0.38	0.21	0.23
mir-262	0.19	0.04	0.19
mir-265	0.11	0.00	0.00
mir-34	2,436.66	2,547.27	2,759.50
mir-35	239.22	242.67	190.16
mir-355	0.04	0.07	0.04
mir-356	0.15	0.04	0.12
mir-359	0.27	0.42	0.08
mir-36	42.75	47.82	43.29
mir-360	0.04	0.04	0.00
mir-37	719.97	787.98	659.22
mir-392	0.27	0.28	0.12
mir-40	79.78	105.43	82.94
mir-42	26.86	36.13	28.49
mir-43	5.01	4.23	4.95
mir-46	0.30	0.38	0.35
mir-47	9.29	13.92	6.88
mir-49	83.27	93.45	89.78
mir-50	625.78	622.21	574.38
mir-53	2.88	5.73	3.05
mir-54	991.97	721.77	545.62
mir-55	1,396.36	1,089.57	989.21
mir-56	853.47	782.67	644.99
mir-57	1,711.18	1,010.31	1,832.21
mir-59	20.18	28.77	21.03
mir-60	27.39	30.85	32.00
mir-61	351.43	244.96	252.97
mir-63	263.12	321.93	286.94

mir-64	248.67	287.78	261.39
mir-65	149.47	242.78	192.17
mir-72	24,746.19	19,737.11	22,788.62
mir-73	16,717.42	14,438.91	14,659.61
mir-74	16.20	26.76	13.88
mir-75	532.38	711.95	560.20
mir-76	5.39	3.68	4.41
mir-77	13.88	26.24	16.50
mir-78	0.00	0.00	0.00
mir-784	1.67	2.71	1.01
mir-785	21.70	19.54	14.15
mir-786	7.28	9.27	4.95
mir-787	22.53	20.96	18.51
mir-788	64.83	46.64	35.17
mir-79	17.64	58.06	52.80
mir-790	7.89	5.21	4.91
mir-791	16.27	12.46	12.06
mir-792	0.65	0.38	0.08
mir-793	18.17	14.05	12.87
mir-794	2.77	2.67	2.94
mir-795	540.50	398.03	498.20
mir-796	40.40	32.72	29.41
mir-797	3.64	2.60	4.48
mir-798	4.06	3.37	1.51
mir-800	0.08	0.28	0.19
mir-84	305.87	302.32	242.84
mir-85	88.54	78.25	41.12
mir-90	197.91	158.21	191.09

Table 2-S4. Salient proteins and peptides identified in CGH-1::GFP mass spectrometry.

Table S4

Mass spectrometry of CGH-1::GFP complexes		
Protein	**Peptide Coverage**	**Spectral Counts**
KIN-3	32.5%	16
KIN-10	30.3%	6
NHL-2	4.5%	2
VIG-1	14.3%	3
AIN-1	0%	0
ALG-1	0%	0
TSN-1	0%	0
CGH-1	32.6%	16

CGH-1::GFP Phospho-Mass spectrometry			
Protein	**Peptide Coverage**	**Spectral Count/Peptide**	**Peptides**
CGH-1	4.4%	3	MSGAEQQQIVPANNGDENWK.A
		4	MSGAEQQQIVPANNGDENWK.A *
		5	SGAEQQQIVPANNGDENWK.A
		3	GAEQQQIVPANNGDENWK.A
		11	AEQQQIVPANNGDENWK.A
		7	EQQQIVPANNGDENWK.A
		7	QQQIVPANNGDENWK.A
		17	IVPANNGDENWK.A
		15	VPANNGDENWK.A
	total spectral count	72	S: phosphorylated amino acid * phosphopeptide not identified in *kin-3* RNAi sample

REFERENCES

1. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75: 843-854.
2. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, et al. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403: 901-906.
3. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136: 215-233.
4. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic acids research 34: D140-144.
5. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome research 19: 92-105.
6. Kim VN (2005) MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 6: 376-385.
7. Kim VN, Han J, Siomi MC (2009) Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10: 126-139.
8. Huntzinger E, Izaurralde E (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nature reviews Genetics 12: 99-110.
9. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, et al. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 106: 23-34.
10. Tops BB, Plasterk RH, Ketting RF (2006) The Caenorhabditis elegans Argonautes ALG-1 and ALG-2: almost identical yet different. Cold Spring Harb Symp Quant Biol 71: 189-194.
11. Ding L, Spencer A, Morita K, Han M (2005) The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Mol Cell 19: 437-447.
12. Zhang L, Ding L, Cheung TH, Dong MQ, Chen J, et al. (2007) Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Mol Cell 28: 598-613.
13. Rajyaguru P, Parker R (2009) CGH-1 and the control of maternal mRNAs. Trends in cell biology 19: 24-28.
14. Boag PR, Atalay A, Robida S, Reinke V, Blackwell TK (2008) Protection of specific maternal messenger RNAs by the P body protein CGH-1 (Dhh1/RCK) during Caenorhabditis elegans oogenesis. The Journal of cell biology 182: 543-557.
15. Hammell CM, Lubin I, Boag PR, Blackwell TK, Ambros V (2009) nhl-2 Modulates microRNA activity in Caenorhabditis elegans. Cell 136: 926-938.
16. Rouya C, Siddiqui N, Morita M, Duchaine TF, Fabian MR, et al. (2014) Human DDX6 effects miRNA-mediated gene silencing via direct binding to CNOT1. RNA 20: 1398-1409.
17. Mathys H, Basquin J, Ozgur S, Czarnocki-Cieciura M, Bonneau F, et al. (2014) Structural and biochemical insights to the role of the CCR4-NOT complex and DDX6 ATPase in microRNA repression. Molecular cell 54: 751-765.

18. Chen Y, Boland A, Kuzuoglu-Ozturk D, Bawankar P, Loh B, et al. (2014) A DDX6-CNOT1 complex and W-binding pockets in CNOT9 reveal direct links between miRNA target recognition and silencing. Molecular cell 54: 737-750.
19. Caudy AA, Ketting RF, Hammond SM, Denli AM, Bathoorn AM, et al. (2003) A micrococcal nuclease homologue in RNAi effector complexes. Nature 425: 411-414.
20. Wilczynska A, Bushell M (2015) The complexity of miRNA-mediated repression. Cell death and differentiation 22: 22-33.
21. Kim YK, Heo I, Kim VN (2010) Modifications of small RNAs and their associated proteins. Cell 143: 703-709.
22. Qi HH, Ongusaha PP, Myllyharju J, Cheng D, Pakkanen O, et al. (2008) Prolyl 4-hydroxylation regulates Argonaute 2 stability. Nature 455: 421-424.
23. Zeng Y, Sankala H, Zhang X, Graves PR (2008) Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. Biochem J 413: 429-436.
24. Rybak A, Fuchs H, Hadian K, Smirnova L, Wulczyn EA, et al. (2009) The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. Nat Cell Biol 11: 1411-1420.
25. Rudel S, Wang Y, Lenobel R, Korner R, Hsiao HH, et al. (2011) Phosphorylation of human Argonaute proteins affects small RNA binding. Nucleic acids research 39: 2330-2343.
26. Sahin U, Lapaquette P, Andrieux A, Faure G, Dejean A (2014) Sumoylation of human argonaute 2 at lysine-402 regulates its stability. PloS one 9: e102957.
27. Wulczyn FG, Cuevas E, Franzoni E, Rybak A (2011) miRNAs Need a Trim : Regulation of miRNA Activity by Trim-NHL Proteins. Advances in experimental medicine and biology 700: 85-105.
28. Schwamborn JC, Berezikov E, Knoblich JA (2009) The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell 136: 913-925.
29. Huang KL, Chadee AB, Chen CY, Zhang Y, Shyu AB (2013) Phosphorylation at intrinsically disordered regions of PAM2 motif-containing proteins modulates their interactions with PABPC1 and influences mRNA fate. RNA 19: 295-305.
30. Shen J, Xia W, Khotskaya YB, Huo L, Nakanishi K, et al. (2013) EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. Nature 497: 383-387.
31. Kim JK, Gabel HW, Kamath RS, Tewari M, Pasquinelli A, et al. (2005) Functional genomic analysis of RNA interference in C. elegans. Science 308: 1164-1167.
32. Tabach Y, Golan T, Hernandez-Hernandez A, Messer AR, Fukuda T, et al. (2013) Human disease locus discovery and mapping to molecular pathways through phylogenetic profiling. Molecular systems biology 9: 692.
33. Niefind K, Raaf J, Issinger OG (2009) Protein kinase CK2 in health and disease: Protein kinase CK2: from structures to insights. Cell Mol Life Sci 66: 1800-1816.
34. Hu E, Rubin CS (1990) Casein kinase II from Caenorhabditis elegans. Properties and developmental regulation of the enzyme; cloning and sequence analyses of cDNA and the gene for the catalytic subunit. J Biol Chem 265: 5072-5080.

35. Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP, et al. (2005) The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in Caenorhabditis elegans. Dev Cell 9: 403-414.
36. Sulston JEaH, H. R. (1977) Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Developmental biology 56: 110-156.
37. Koh K, Rothman JH (2001) ELT-5 and ELT-6 are required continuously to regulate epidermal seam cell differentiation and cell fusion in C. elegans. Development 128: 2867-2880.
38. Liu Z, Kirch S, Ambros V (1995) The Caenorhabditis elegans heterochronic gene pathway controls stage-specific transcription of collagen genes. Development 121: 2471-2478.
39. Abrahante JE, Miller EA, Rougvie AE (1998) Identification of heterochronic mutants in Caenorhabditis elegans. Temporal misexpression of a collagen::green fluorescent protein fusion gene. Genetics 149: 1335-1351.
40. Hayes GD, Frand AR, Ruvkun G (2006) The mir-84 and let-7 paralogous microRNA genes of Caenorhabditis elegans direct the cessation of molting via the conserved nuclear hormone receptors NHR-23 and NHR-25. Development 133: 4631-4641.
41. Ecsedi M, Rausch M, Grosshans H (2015) The let-7 microRNA directs vulval development through a single target. Developmental cell 32: 335-344.
42. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, et al. (2005) RAS is regulated by the let-7 microRNA family. Cell 120: 635-647.
43. Sternberg PW (2005) Vulval development. WormBook: 1-28.
44. Eisenmann DM, Kim SK (1997) Mechanism of activation of the Caenorhabditis elegans ras homologue let-60 by a novel, temperature-sensitive, gain-of-function mutation. Genetics 146: 553-565.
45. Johnston RJ, Hobert O (2003) A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature 426: 845-849.
46. Kennedy S, Wang D, Ruvkun G (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in C. elegans. Nature 427: 645-649.
47. Schmitz C, Kinge P, Hutter H (2007) Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive Caenorhabditis elegans strain nre-1(hd20) lin-15b(hd126). Proc Natl Acad Sci U S A 104: 834-839.
48. Sarin S, O'Meara MM, Flowers EB, Antonio C, Poole RJ, et al. (2007) Genetic screens for Caenorhabditis elegans mutants defective in left/right asymmetric neuronal fate specification. Genetics 176: 2109-2130.
49. Alvarez-Saavedra E, Horvitz HR (2010) Many families of C. elegans microRNAs are not essential for development or viability. Curr Biol 20: 367-373.
50. Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, et al. (2000) The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell 5: 659-669.
51. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, et al. (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 122: 553-563.
52. Lin YC, Hsieh LC, Kuo MW, Yu J, Kuo HH, et al. (2007) Human TRIM71 and its nematode homologue are targets of let-7 microRNA and its zebrafish orthologue is essential for development. Molecular biology and evolution 24: 2525-2534.

53. Ding XC, Grosshans H (2009) Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins. EMBO J 28: 213-222.
54. Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75: 855-862.
55. Simon DJ, Madison JM, Conery AL, Thompson-Peer KL, Soskis M, et al. (2008) The microRNA miR-1 regulates a MEF-2-dependent retrograde signal at neuromuscular junctions. Cell 133: 903-915.
56. Chan SP, Slack FJ (2009) Ribosomal protein RPS-14 modulates let-7 microRNA function in Caenorhabditis elegans. Dev Biol 334: 152-160.
57. Zielinska DF, Gnad F, Jedrusik-Bode M, Wisniewski JR, Mann M (2009) Caenorhabditis elegans has a phosphoproteome atypical for metazoans that is enriched in developmental and sex determination proteins. J Proteome Res 8: 4039-4049.
58. Yochem J, Greenwald I (1989) glp-1 and lin-12, genes implicated in distinct cell-cell interactions in C. elegans, encode similar transmembrane proteins. Cell 58: 553-563.
59. Austin J, Kimble J (1987) glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans. Cell 51: 589-599.
60. Dosztanyi Z, Csizmok V, Tompa P, Simon I (2005) IUPred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. Bioinformatics 21: 3433-3434.
61. Collins MO, Yu L, Campuzano I, Grant SG, Choudhary JS (2008) Phosphoproteomic analysis of the mouse brain cytosol reveals a predominance of protein phosphorylation in regions of intrinsic sequence disorder. Molecular & cellular proteomics : MCP 7: 1331-1348.
62. Scheckel C, Gaidatzis D, Wright JE, Ciosk R (2012) Genome-wide analysis of GLD-1-mediated mRNA regulation suggests a role in mRNA storage. PLoS genetics 8: e1002742.
63. Cassada RC, Russell RL (1975) The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental biology 46: 326-342.
64. Liu ZC, Ambros V (1991) Alternative Temporal Control-Systems for Hypodermal Cell-Differentiation in Caenorhabditis-Elegans. Nature 350: 162-165.
65. Euling S, Ambros V (1996) Reversal of cell fate determination in Caenorhabditis elegans vulval development. Development 122: 2507-2515.
66. Karp X, Ambros V (2012) Dauer larva quiescence alters the circuitry of microRNA pathways regulating cell fate progression in C. elegans. Development 139: 2177-2186.
67. Poole A, Poore T, Bandhakavi S, McCann RO, Hanna DE, et al. (2005) A global view of CK2 function and regulation. Mol Cell Biochem 274: 163-170.
68. Stiernagle T (2006) Maintenance of C. elegans. WormBook: 1-11.
69. Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, et al. (2000) Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature 408: 325-330.

70. Rual JF, Ceron J, Koreth J, Hao T, Nicot AS, et al. (2004) Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. Genome Res 14: 2162-2168.
71. Kamath RS, Ahringer J (2003) Genome-wide RNAi screening in Caenorhabditis elegans. Methods 30: 313-321.
72. Harper S, Speicher DW (2011) Purification of proteins fused to glutathione S-transferase. Methods in molecular biology 681: 259-280.
73. Reinhart BJ, Ruvkun G (2001) Isoform-specific mutations in the Caenorhabditis elegans heterochronic gene lin-14 affect stage-specific patterning. Genetics 157: 199-209.
74. Zanin E, Dumont J, Gassmann R, Cheeseman I, Maddox P, et al. (2011) Affinity purification of protein complexes in C. elegans. Methods in cell biology 106: 289-322.
75. Gu W, Shirayama M, Conte D, Jr., Vasale J, Batista PJ, et al. (2009) Distinct argonaute-mediated 22G-RNA pathways direct genome surveillance in the C. elegans germline. Mol Cell 36: 231-244.
76. Billi AC, Alessi AF, Khivansara V, Han T, Freeberg M, et al. (2012) The Caenorhabditis elegans HEN1 ortholog, HENN-1, methylates and stabilizes select subclasses of germline small RNAs. PLoS genetics 8: e1002617.
77. Pall GS, Codony-Servat C, Byrne J, Ritchie L, Hamilton A (2007) Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. Nucleic Acids Res 35: e60.
78. Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nature Protocols 1: 1559-1582.
79. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nature methods 9: 357-359.
80. Mello CC, Kramer JM, Stinchcomb D, Ambros V (1991) Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J 10: 3959-3970.
81. Vowels JJ, Thomas JH (1994) Multiple chemosensory defects in daf-11 and daf-21 mutants of Caenorhabditis elegans. Genetics 138: 303-316.