KH domain containing RNA-binding proteins coordinate with microRNAs to regulate Caenorhabditis elegans development

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Abstract

MicroRNAs (miRNAs) and RNA-binding proteins (RBPs) regulate gene expression at the post-transcriptional level, but the extent to which these key regulators of gene expression coordinate their activities and the precise mechanisms of this coordination are not well understood. RBPs often have recognizable RNA binding domains that correlate with specific protein function. Recently, several RBPs containing K homology (KH) RNA binding domains were shown to work with miRNAs to regulate gene expression, raising the possibility that KH domain proteins functionally interact with miRNAs during Caenorhabditis elegans development, we knocked down twenty-four genes encoding KH-domain proteins in several miRNA sensitized genetic backgrounds. Here, we report that a majority of the KH domain-containing genes genetically interact with multiple miRNAs and Argonaute alg-1. Interestingly, two KH domain genes, predicted splicing factors sfa-1 and asd-2, genetically interacted with all of the miRNA mutants tested, whereas other KH domain genes showed genetic interactions only with specific miRNAs. Our domain architecture and phylogenetic relationship analyses of the C. elegans KH domain-containing proteins revealed potential groups that may share structure and function. Collectively, we show that many C. elegans KH domain RBPs functionally interact with miRNAs, suggesting direct or indirect coordination between these two classes of post-transcriptional gene expression regulators.

Keywords: microRNA; RNA-binding protein; KH domain; hnRNPK

Introduction

Most developmental and cellular processes rely on precise choreography of gene regulatory networks that incorporate a wide range of cellular and environmental inputs. Evolution of multiple regulatory pathways provided cells with multifaceted and combinatorial methods of regulating gene expression allowing for robustness, flexibility, and rapid remodeling of expression patterns. One of the essential layers of gene regulation occurs at the post-transcriptional level and is effected by two classes of molecules: small noncoding RNAs called microRNAs (miRNAs) and RNA-binding proteins (RBPs). The human genome is predicted to encode more than 180 miRNAs (Ambros and Ruvkun 2018) and at least 850 RBPs (Gerstberger et al. 2019). Pri-miRNAs are then processed by consecutive enzymatic interactions only with specific miRNAs. Our domain architecture and phylogenetic relationship analyses of the C. elegans KH domain-containing proteins revealed potential groups that may share structure and function. Collectively, we show that many C. elegans KH domain RBPs functionally interact with miRNAs, suggesting direct or indirect coordination between these two classes of post-transcriptional gene expression regulators.

Keywords: microRNA; RNA-binding protein; KH domain; hnRNPK

Most mature miRNAs are generated via a canonical multi-step biogenesis pathway that starts with transcription of primary miRNA (pri-miRNA) transcripts (reviewed in Gebert and MacRae 2019). Pri-miRNAs are then processed by consecutive enzymatic activities of Drosha and Dicer endonucleases to generate a double-stranded RNA duplex, which is ultimately loaded into an Argonaute protein. A single miRNA strand is retained by an Argonaute and the mature miRNA silencing complex (miRISC) is formed when the miRNA-loaded Argonaute associates with a GW182 effector on the target messenger RNA (mRNA) (reviewed in Gebert and MacRae 2019). The miRISC identifies target mRNAs through partial sequence complementarity, ultimately resulting in translation repression and/or mRNA degradation (reviewed in O’Brien et al. 2018; Gebert and MacRae 2019).

RNA-binding proteins regulate diverse aspects of mRNA life cycle, including splicing, transport, and stability (Dassi 2017). Diversity in protein architecture and auxiliary domains, as well as a high degree of modularity, allows RBPs to impart specific and potent effects on the gene expression of their targets (Janga 2012). For example, the PUF family of proteins in C. elegans inhibits translation of their mRNA targets through sequence-specific binding of the 3’UTR in order to promote deadenylation or by physically blocking cap recognition by translation initiation factors (reviewed in Wang and Voronina 2020). Other proteins like OMA-1 appear to play a more nuanced role by concomitantly binding the 3’UTRs of mRNAs along with translational repressors like LIN-41 to mediate the selective repression-to-activation
transition for a subset of mRNAs essential for oogenesis (Tsukamoto et al. 2017). Here, RBPs and miRNAs are thought to cooperatively interact, and de-regulation of their activity can precipitate widespread disruption of gene regulatory networks resulting in a variety of cell pathologies and disease states (Tufekci et al. 2013; O’Brien et al. 2018).

To effect post-transcriptional regulation of gene expression, RBP and miRNA activity can intersect on multiple levels. On a most basic level, miRNA biogenesis is performed and aided by RBPs (reviewed in Gebert and MacRae 2019). RBPs may directly associate with miRNA-target complexes to modulate the downstream effects on target gene expression (Hammell et al. 2009; Schwamborn et al. 2009; Wu et al. 2017). Coordination between RBPs and miRNAs can also be indirect, with individual factors affecting the target mRNA in distinct ways, ultimately resulting in a unique combinatorial gene regulatory outcome.

Among RBPs identified as modulators of miRNA activity are three proteins that share a conserved RNA-binding K-homology (KH) domain (Akay et al. 2013; Zabinski et al. 2017; Li et al. 2019). KH domain was first described in human hnRNP K (Siomi et al. 1993, reviewed in Geuen et al. 2016) and is present alone or in tandem in a large group of RBPs associated with transcription or translation regulation (Nicastro et al. 2015; Domínguez et al. 2018). The type I KH domain, found in eukaryotes, is approximately 70 amino acids and is characterized by three-parallel beta-sheets abutted by three alpha-helices; it includes the GXXG loop, which is thought to be responsible for nucleic acid binding (Grishin 2001; Valverde et al. 2008). We recently showed that HRPK-1, a KH domain-containing protein, physically and functionally interacts with miRNA complexes to modulate gene expression during proliferation and development (Li et al. 2017). VGLN-1 binds miRNAs rich with miRNA binding sites in their 3’UTR (Zabinski et al. 2017) and may serve as a platform, bridging interactions between multiple miRNAs, and proteins to regulate gene expression (Zabinski et al. 2017). GLD-1, an RNA-binding protein and a well-characterized translational repressor that regulates germline development (Marin and Evans 2003), has been shown to genetically interact with multiple miRNAs (Akay et al. 2013). GLD-1 contains a single KH domain, functionally interacts with miRNA modulators, nhl-1 and vgl-1, and physically interacts with ALG-1, CGH-1, and PAB-1, proteins that are key for miRNA gene regulatory activity (Akay et al. 2013). Collectively, these findings suggest that RBPs that harbor KH domain(s) may be functionally important for miRNA-dependent gene regulation.

To determine the extent of functional coordination between the KH domain-containing proteins and miRNAs, we knocked down 24 additional predicted C. elegans KH domain genes in sensitized miRNA genetic backgrounds. Strikingly, knockdown of 18 KH domain genes resulted in a modulation of a phenotype associated with a partial loss of miRNA activity. We found that several genes, including the predicted splicing factors sfa-1 and osd-2, genetically interacted with multiple miRNA families, suggesting that splicing events may influence miRNA gene regulatory activity. Other genes, such as Y69A2AR.32, showed miRNA family specificity. Knockdown of most KH domain genes resulted in enhancement of miRNA reduction-of-function phenotypes, suggesting a normally positive functional interaction between KH domain RBPs and miRNAs. However, knockdown of several genes resulted in mild to strong suppression of defects observed in an Argonaute alg-1 antimorphic mutant, suggesting that some of these factors normally act antagonistically to miRNAs. Overall, this work provides a comprehensive examination of the genetic interactions between miRNAs and KH domain RBPs in C. elegans, presents a phylogenetic and a domain analysis of C. elegans KH domain-containing proteins, and suggests that these RBPs may directly or indirectly coordinate with miRNA pathways to regulate gene expression.

Materials and methods

Worm strains

Worm culture and maintenance was performed as previously described (Brenner 1974). Bristol N2 was used as the wild-type strain. Strains used in this study are OH3646 let-6(ot153); ots114 [Plom-6-gfp + rol-6(sv1006)]; OH128 ots114 [Plom-p-gfp + rol-6(sv1006)]; P53662 sy1563 [cog-1::gfp + unc-119(+)]; OH370 ots193 [Pocq-1::lsy-6 + rol-6(sv1006)]; sy1s63, VT1296 mir-48 mir-241(nDf51) col-19::gfp (maIs105), MT7626 let-7(n2853), VT2223 lin-31(n1053); col-19::gfp (maIs105); alg-1(ma202), HW113 [Pdp-30::GFP(PEST)-H2B:lin-41 3’UTR (xelS78); Pdp-30::mcherry::H2B:: artificial 3’UTR (xelS36)]; HW1114 [Pdp-30::GFP(PEST)-H2B:lin-41 3’UTR (xelS78); Pdp-30::mcherry::H2B:: artificial 3’UTR (xelS36); let-7(n2853)]; HW1159 [Pdp-30::GFP(PEST)-H2B:lin-41 delta LCS 3’UTR (xelS37); Pdp-30::mcherry::H2B:: artificial 3’UTR (xelS36)], and BW1992 hbl-1::gfp::NLS::hbl-1 3’UTR (ctIs39). All strains were grown at 20°C with the exception of MT7626 let-7(n2853) which was grown and maintained at 15°C to prevent excess bursting.

RNA interference

RNAi constructs (pL4440) were obtained from the Ahringer RNAi library (Kamath et al. 2000; Source Biosciences) except for bcc-1 and E029D1.1 which were obtained from the Vidal RNAi library (Rual et al. 2004; Source Biosciences). In addition, 3 RNAi clones were constructed by genomic amplification of the endogenous loci and cloning of the fragment into the L4440 vector. The fubl-3 clone was generated by using forward 5’-GCCCACTAGTGGAC TAACTGCAACGTTCAA-3’ and reverse 5’-GGCCAGATCTC GTCTCGGGAGGAAAG-3’. The fubl-3 clone was generated using forward 5’-GCTCAAGATCTGCGCAGTTCATGGCCAAAC-3’ and reverse 5’-GTAAGTACGGGAATCTTCCCTTCTCACA-3’. The B280.17 clone was generated using forward 5’-GCCCAAGATCTCTTCATGTCGTTTCA-3’ and reverse 5’-ATAGGTCACGGCGCACTGTCGGAGAAAG-3’. The amplified genomic fragments containing restriction sites were digested using SpeI and KpnI (fubl-3) or BglII and KpnI (Y69A2AR.32 and B280.17) and were ligated with the digested L4440 vector using NEB (M2200) Quick Ligation protocol. Ligated plasmid was then transformed into E. coli HT115 bacteria. Sequence insertion into the L4440 plasmid was confirmed via Sanger sequencing (using M13 forward sequencing primer). Although the Ahringer clone targeting mex-3 was obtained, RNAi of mex-3 in lsy-6(ot150) and mir-48 mir-241(nDf51) resulted in highly penetrant embryonic lethality preventing scoring of the F1 progeny of the RNAi treated animals.

RNAi experiments were done by feeding and performed at 20°C unless otherwise stated and as described below. All RNAi experiments of individual genes were performed in parallel with empty vector RNAi (negative control). dcr-1 RNAi was used as a positive control as loss or reduction of dcr-1 eliminates/impaits miRNA biogenesis. RNAi plates were prepared and seeded using standard methods (Kamath et al. 2000). Scoring requiring fluorescence was done on a Leica DM6B fluorescent compound microscope. Imaging of fluorescence-based phenotypes was done using the Leica DM6B mounted camera and processed using Leica.
software. Photoplates were assembled using Adobe Illustrator. Scoring of vulval bursting, brood size, and embryonic lethality were done a standard Leica dissecting microscope. The number of animals scored per replicate as well as the percentage of animals displaying the abnormal phenotype in each replicate are reported in Supplementary Tables S1 and S2.

Despite the overall relatedness of protein architecture among member of phylogenetic clades (Figure 8), BLAST (NCBI) searches for RNAi-targeted regions suggest there is sufficient variation in nucleotide sequence for individual RNAi clones to specifically target the gene of choice. The rare exceptions may be the fubi genes, and the ass-2/gid-1 pair which show a very low level of overlap in targeted sequence, allowing for the possibility that some cross-gene RNAi targeting may occur.

**ASEL cell fate differentiation**

*Plim-6::gfp (ats114) and lsy-6(ot150); Plim-6::gfp (ats114) worms were placed on RNAi as embryos and F1 progeny were scored as L4 or young adults to increase the ease of detecting fluorescent signal in ASEL neurons. Each group of genes was scored alongside the negative control (empty L4440 vector) and our positive control (dcr-1 RNAi). Worms were scored as cell fate defective when* plim-6::gfp *was undetectable in the ASEL neuron soma.

**Uterine cog-1::gfp**

cog-1::gfp (syls63) and cog-1::gfp (syls63); ats193[Pcoag-1::lsy-6; rol-6(su1006)] worms were placed on RNAi as embryos and F1 progeny were scored at mid-late L4s in order to ensure a strong GFP signal in both vulval and uterine cells. Each group of genes was scored alongside the negative control (empty L4440 vector) and our positive control (dcr-1 RNAi). Worms were considered to have abnormal uterine cog-1::gfp if either the anterior or posterior or both uterine cells were lacking GFP. cog-1 expression was scored as normal when GFP expression was observed in both uterine cells and in vulval cells.

**Hydropodermal col-19::gfp expression and seam cell number**

col-19::gfp (maIs105) and mir-48 mir-241(nDf51) col-19::gfp (maIs105) animals were placed on RNAi as young L4s and their F1 progeny were scored as young adults. Each group of genes was scored alongside the negative control (empty L4440 vector) and our positive control (dcr-1 RNAi). Worms were scored first for the presence of col-19::gfp in the hypodermal cells. Normal expression was defined as all hypodermal cells expressing col-19::gfp, whereas abnormal expression was defined as GFP signal absent in many or all of hypodermal cells. Worms were also scored for the number of seam cells present between the pharynx and rectal cells; seam cells were identified using the col-19::gfp transgene. lin-31(n1053), col-19::gfp (maIs105), alq-1(ma202) worms were scored in an identical manner when assaying hypodermal col-19::gfp expression.

**hbl-1::gfp expression**

*hbl-1::gfp::NLS:hbl-1 3’UTR (ctIs39) animals were synchronized by bleaching and plated on RNAi plates with the RNAi bacteria supplemented with fluorescent beads for accurate staging (Nika et al. 2016). Worms were grown until the majority began the L2 molt, at which point worms were screened for the presence of fluorescent beads within the gut. Worms that lacked beads and therefore had entered the L2 molt were picked to a new plate seeded with the equivalent RNAi bacteria without beads. The molting worms were then screened every 30 minutes for resumption of pumping indicating they had exited the molt into L3. The worms were then scored at 40X magnification for hbl-1::gfp expression in hypodermal cells. Representative images were taken at 63X magnification.**

**Vulval bursting**

let-7(n2853) worms were grown and maintained at 15°C. Embryos were synchronized by hypochloride/NaOH solution and embryos plated directly onto RNAi plates as previously described (Parry et al. 2007). The embryos were hatched and grown at 15°C until young adults. Worms were scored for vulval bursting (~6h after the L4 molt to ensure all animals had reached adulthood. Each group of genes was scored alongside the negative control (empty L4440 vector) and our positive control (dcr-1 RNAi).

**lin-41 reporter assay**

[Pdpy-30::GFP(PEST)-H2B::lin-41 3’UTR (xeSi78), Pdpy-30::mCherry::H2B::artificial 3’UTR (xeSi36)] (HW1113), [Pdpy-30::GFP(PEST)-H2B::lin-41 3’UTR (xeSi78), Pdpy-30::mCherry::H2B::artificial 3’UTR (xeSi36); let-7(n2853)] (HW1114), [Pdpy-30::GFP(PEST)-H2B::lin-41 delta LSC 3’UTR (xeSi87), Pdpy-30::mCherry::H2B::artificial 3’UTR (xeSi36)] (HW1159) (Ecsedi et al. 2015) animals were synchronized by bleaching. Embryos were plated onto seeded RNAi plates. Worms were grown until L4 at which point they were imaged at 63X magnification in red and green channels to capture GFP and mCherry expression in the vulval cells. Leica image analysis software was used to determine the fluorescence in each region of interest (ROI) surrounding each of six vulval cells in both red and green channels. Identical exposure and microscope settings were used for all imaging to allow quantification and comparison of signals. To quantify the changes in *lin-41* expression we divided the relative signal intensity of the green channel by the signal intensity in the red channels in each of the vulval cells. The average signal intensity for the vulval tissue was determined by averaging the signal ratios across the six cells scored for each worm. The representative images were adjusted for brightness and contrast post-quantification to allow the reader to more easily observe the fluorescence in cells of interest.

**Brood size and embryonic lethality**

Wildtype (N2) worms were placed on RNAi as L4s and allowed to lay embryos. When the F1 progeny reached the L4 stage, individual hermaphrodites were moved to their own RNAi plates and allowed to lay embryos for 24 h. After 24 h, each animal was moved to a fresh RNAi plate each day for three additional days. Live larvae were counted on each plate (by picking) 24 and 48 h after the parent has been moved to ensure all larvae were counted. Dead embryos on each plate were counted 48 h after removal of the parent. The total number of live larvae and dead embryos for each hermaphrodite was tallied and together encompass brood size. Embryonic lethality was calculated as (# dead embryos/total brood size) × 100%. Larval arrest was rarely seen, but when it did occur these worms were counted as “live larvae” because they had successfully hatched and developed beyond the embryonic stage.

**Phylogenetic analysis**

Full proteins sequences of the longest isoforms for each protein were collected from Wormbase and entered to the Mega X program. A MUSCLE protein alignment was carried out to provide input for further phylogenetic analysis. In order to construct the tree, we selected the Maximum Likelihood method and
bootstraped the tree-building (1000 iterations) to increase the stringency of the method. A simple LG model was selected for the substitution model, using a Nearest-Neighbor-Interchange (NNI) method. The phylogenic tree shown represents 27 of the 28 KH domain proteins in the C. elegans genome: mask-1 was excluded due to extensive length and sequence/domain variability from the rest of the protein family.

**Protein domain and architecture**

To generate the protein domain graphics, we first determined the longest isoform of each individual protein. The amino acid sequence of the proteins were obtained from Wormbase.org and entered into Simple Modular Architecture Research Tool (SMART) (Letunic and Bork 2017) under the Genomic options. Domain start and end points were noted and used to generate the protein graphics in Adobe Illustrator.

To generate the coverage of each RNAi clone used in this study, primer pairs were obtained from the Ahringer library database, aligned to the appropriate transcript. Each RNAi target was then translated in the appropriate frame and aligned to the complete protein sequence. Predicted NLS sites were determined using cNLS Mapper using a threshold of 5.0 (Kosugi et al. 2009). Only high confidence (score > 8.0) NLS regions were included in the domain graphics.

**Statistical analysis**

All statistics were done using GraphPad Prism software. Statistical significance was determined using a one-way ANOVA test. To make the desired comparisons and avoid the loss of statistical power inherent to multiple comparisons, we used planned comparisons to compare each individual gene RNAi with vector control RNAi. Bonferroni correction was applied as a post hoc analysis.

**Data availability**

Strains and plasmids are available upon request. All data necessary for confirming the finding of this article are present within the article and the associated figures, and tables. Supplementary material is available at figshare: https://doi.org/10.25387/g3.13551626.

**Results**

**Multiple KH domain genes genetically interact with lsy-6 miRNA in ASEL neuronal cell fate specification**

The lsy-6 miRNA controls cell fate specification of the ASEL/ASER sensory neuron pair. lsy-6 normally represses expression of cog-1 in the ASEL neuron, ultimately resulting in an ASER specific gene expression pattern (Johnston and Hobert 2003) (Figure 1A). Loss of lsy-6 activity results in an inappropriate cell fate switch of the ASEL neuron to the ASER cell fate (Johnston and Hobert 2003). The lsy-6(ot150) reduction-of-function mutation causes a low penetrance phenotype, with ~15% of lsy-6(ot150) animals displaying an ASER cell fate defective phenotype. This cell fate defect can be observed by the loss of the Plim-6::gfp expression within the ASEL neuron (Figure 1A and B).

To identify whether KH-domain genes play a role in lsy-6-dependent neuronal cell fate specification, we knocked down twenty-four KH domain genes in the lsy-6(ot150) mutant background (Figure 1B) and assayed the penetrance of the ASEL cell fate defect. Knockdown of five of the KH domain genes, pes-4, sfu-1, mask-1, F54D1.1, and asd-2 significantly enhanced the lsy-6(ot150) cell fate defective phenotype (Figure 1B and Table 1). RNAi-mediated knockdown of the KH domain genes did not result in a phenotype in the absence of the lsy-6(ot150) allele, with the exception of F54D1.1 and mxt-1, whose depletion caused an occasional loss of Plim-6::gfp expression in ASEL (Figure 1B). Furthermore, RNAi of T10E9.14, fubl-4, tofu-7 and pno-1 resulted in variable and/or mild but not statistically significant enhancement of the lsy-6(ot150) phenotype (Figure 1B).

**KH domain genes coordinate with lsy-6 to regulate the expression of cog-1**

Next, we wanted to determine whether the genes that genetically interacted with lsy-6(ot150) were also able to regulate a lsy-6 target, cog-1 (Johnston and Hobert 2003). Although lsy-6 expression is normally restricted to neuronal tissues, its endogenous target cog-1 is more broadly expressed (Palmer et al. 2002). Expression of lsy-6 from the cog-1 promoter represses the cog-1::gfp reporter in the uterine and vulval cells (Johnston and Hobert 2003) (Figure 2A). Therefore, we can use the lsy-6-mediated repression of cog-1 to assay the effects of knocking down potential modulators of lsy-6 activity. Indeed, RNAi of five genes, sfu-1, tofu-7, pes-4, asd-2, and T10E9.14 resulted in a significant de-repression of cog-1::gfp expression in uterine cells (Figure 2B and Table 1), suggesting that these genes may coordinate with lsy-6 in repressing cog-1. Although not statistically significant the knockdown of tofu-7, asd-2, and F54D1.1 mildly repressed cog-1::gfp expression in the uterine cells in the absence of Pog-1::lsy-6 (Figure 2B), suggesting these genes may regulate cog-1 independently of lsy-6. In fact, tofu-7, asd-2, and F54D1.1 may have a more complex functional relationship, perhaps regulating cog-1 through multiple genetic pathways, including one that involves the lsy-6 miRNA. Here, tofu-7, asd-2, and F54D1.1 may act to promote cog-1::gfp expression in the absence of lsy-6, whereas the addition of lsy-6 changes the functional relationship from positive to repressive or may de-repress target gene expression in either direction (Figure 2B).

**KH domains proteins coordinate with let-7 family of miRNAs**

To determine whether KH-domain-containing proteins might coordinate with additional miRNAs beyond lsy-6, we looked for a genetic interaction between the KH domain genes and the let-7 family of miRNAs. The let-7 miRNA family, as part of a complex genetic network, regulates division patterns and terminal cell differentiation of seam cells during C. elegans larval development (Reinhart et al. 2000; Slack et al. 2000; Abbott et al. 2005). Three members of the let-7 family, mir-48, mir-241, and mir-84 act redundantly to control seam cell divisions by inhibiting the proliferative divisions of the L2 stage and promoting the self-renewing seam cell divisions of the L3 stage (Abbott et al. 2005). Loss of mir-48, mir-241, and mir-84 leads to a highly penetrant reiteration of the proliferative L2 seam cell division leading to increased seam cell number, delayed alae formation, and delayed expression of the adult specific reporter, col-19::gfp (Abbott et al. 2005). Deletion of mir-48 and mir-241, which leaves mir-84 intact, results in milder heterochronic phenotypes including increased seam cell number and delayed alae formation (Abbott et al. 2005).

We performed RNAi for the twenty-four KH domain genes in the mir-48 mir-241(nDf51) mutant background and assayed both col-19::gfp expression and seam cell number in young adult animals (Figure 3, A and B). mir-48 mir-241(nDf51) young adults fail to properly undergo the adult-specific developmental program, thereby showing a delay in col-19::gfp expression consistent with a delay in normal developmental timing. RNAi of thirteen KH
domain genes significantly enhanced the abnormal col-19::gfp expression phenotype observed in mir-48 mir-241(nDf51) animals (Figure 3B and Table 1). RNAi of the twenty-four genes did not produce a phenotype in the absence of the mir-48 mir-241 deletion (Figure 3B), with the exception of B0280.17 RNAi, which showed a very mild defect in hypodermal col-19::gfp expression. In addition, F54D1.1 RNAi produced a mildly penetrant abnormal col-19::gfp expression, but did not enhance the mir-48 mir-241 phenotype to a statistically significant level (Figure 3B). RNAi of nine KH domain genes produced a significant increase in the average number of seam cells in the mir-48 mir-241(nDf51) mutants compared to the empty vector control (Figure 3C and Table 1). Overall, depletion of seven genes both enhanced the delayed hypodermal col-19::gfp expression and increased the seam cell number of mir-48 mir-241(nDf51) mutants (Figure 3, B and C, and Table 1). Together these data suggest that a subset of KH domain genes may coordinate, directly or indirectly, with the let-7 family miRNAs to regulate their target gene expression.

To further explore this level of coordination, we examined the role of KH domain genes in the regulation of hbl-1, a transcription factor and a known target of the let-7 family of miRNAs (Abrahante et al. 2003; Lin et al. 2003; Abbott et al. 2005). Expression of hbl-1 is normally temporally restricted to embryo-L2 animals and upon exit from the L2/L3 molt the expression of hbl-1 is greatly reduced (Abbott et al. 2005). To understand how KH domain genes may be effecting hbl-1 expression, either through the let-7 family of miRNAs or independently, we performed RNAi of the top ten genes identified in the mir-48 mir-241 assays as well as F54D1.1 and assessed hbl-1::gfp expression. Normally, hypodermal hbl-1::gfp becomes downregulated as the animals molt from L2 to L3 and is largely absent in L3 animals (Figure 4A). Since reduction of miRNA activity results in inappropriate hypodermal hbl-1::gfp expression at the L3 stage, we sought to determine the percentage of worms displaying abnormal hypodermal hbl-1::gfp expression in early/mid L3 animals (Figure 4A). RNAi of asd-2, C41G7.3, and sfa-1 produced a significant change in the abnormal expression of hbl-1::gfp in L3 animals, although most genes tested increased the abnormal expression in some RNAi replicates (Figure 4B). These data suggest that KH domain genes may play a role in the regulation of hbl-1, perhaps through the let-7 family of miRNAs or through another indirect mechanism.

To assess the functional relevance of KH domain genes to miRNA activity later in development, we asked whether reducing KH domain gene function impacts activity of let-7 itself. let-7 governs the terminal seam cell differentiation during the transition
Table 1 KH domain genes functionally interact with miRNA sensitized mutants

| Gene or allele | Isy-6(ot150) | Isy-6 | mir-48 mir-241(nDf51) | let-7(n2853) | alg-1(ma202) |
|---------------|-------------|-------|---------------------|-------------|--------------|
| Assay RNAI    | Cell fate* defective | Uterine* cog-1::gfp | Abnormal* col-19::gfp | Seamed* Cell number | Bursting* | Wildtype* col-19::gfp |
| Empty vector  | 15.2 ± 3.9 | 48.8 ± 1.5 | 11.2 ± 2.9 | 13.4 ± 1.4 | 13.7 ± 6.9 | 0 |
| dcr-1         | 34.9 ± 8.6 | 60.2 ± 10.8 | 79.4 ± 15.7 | 14.6 ± 1.9 | 33.4 ± 4.8 | n.d. |
| fub-3         | 17.4 ± 1.5 | n.d.* | 50.4 ± 19.2 | 14.2 ± 1.7 | 31.2 ± 8.7 | 0 |
| fub-4         | 20.8 ± 4.7 | 46.6 ± 17.7 | 45.7 ± 7.5 | 14.1 ± 2.1 | 14.5 ± 10.6 | 0 |
| fub-1         | 19.3 ± 0.0 | n.d. | 37.2 ± 29.5 | 13.3 ± 1.4 | 24.2 ± 6.2 | 2.7 ± 3.8 |
| fub-2         | 19.7 ± 3.1 | n.d. | 22.3 ± 19.7 | 13.2 ± 1.5 | 12.2 ± 2.5 | 2.3 ± 3.2 |
| imph-1        | 14.8 ± 4.2 | n.d. | 6.2 ± 5.4 | 13.5 ± 1.5 | 9.5 ± 1.8 | 7.3 ± 7.2 |
| pes-4         | 33.4 ± 8.0 | 66.2 ± 7.4 | 34.0 ± 12.2 | 12.7 ± 1.1 | 10.6 ± 2.1 | 0 |
| T10E9.14      | 21.4 ± 2.5 | 63.9 ± 3.9 | 51.9 ± 18.9 | 14.1 ± 1.9 | 15.9 ± 8.3 | 0 |
| nova-1        | 14.7 ± 6.3 | n.d. | 6.7 ± 9.4 | 14.0 ± 1.6 | 11.5 ± 4.8 | 3.0 ± 4.2 |
| mxt-1         | 14.5 ± 4.5 | n.d. | 26.6 ± 14.7 | 14.2 ± 1.9 | 28.4 ± 11.9 | 0 |
| asc-1         | 16.1 ± 5.1 | n.d. | 18.1 ± 13.5 | 13.2 ± 1.3 | 15.2 ± 7.1 | 4.2 ± 5.9 |
| akap-1        | 17.3 ± 3.4 | n.d. | 35.8 ± 2.1 | 14.1 ± 1.5 | 13.2 ± 9.4 | 7.2 ± 10.1 |
| tof-7         | 20.6 ± 9.6 | 71.6 ± 1.1 | 37.2 ± 18.2 | 14.2 ± 2.1 | 17.5 ± 6.5 | 0 |
| C06G4.1       | 15.7 ± 4.3 | n.d. | 13.9 ± 4.0 | 13.4 ± 1.6 | 10.9 ± 6.9 | 2.3 ± 3.2 |
| E02D9.1       | 18.2 ± 5.5 | n.d. | 3.9 ± 6.8 | 13.2 ± 1.2 | 7.2 ± 5.4 | 58.0 ± 19.0 |
| sfa-1         | 32.0 ± 2.5 | 73.0 ± 9.0 | 43.9 ± 27.3 | 14.1 ± 1.8 | 25.3 ± 19.2 | 7.2 ± 6.2 |
| ass-2         | 23.2 ± 5.8 | 65.4 ± 1.7 | 51.8 ± 23.9 | 13.9 ± 1.9 | 30.6 ± 19.8 | 0 |
| B0280.17      | 19.3 ± 1.5 | n.d. | 29.9 ± 19.8 | 13.5 ± 1.5 | 31.5 ± 9.2 | 0 |
| F54D1.1       | 23.5 ± 10.5 | 58.3 ± 25.1 | 24.9 ± 7.3 | 13.9 ± 1.4 | 15.7 ± 7.0 | 0 |
| K07H8.9       | 14.7 ± 2.9 | n.d. | 10.8 ± 14.3 | 13.7 ± 1.4 | 9.7 ± 5.4 | 3.0 ± 4.1 |
| Y69A2AR.32    | 18.8 ± 9.1 | n.d. | 40.8 ± 4.6 | 13.5 ± 1.4 | 12.1 ± 3.0 | 6.0 ± 1.6 |
| bcc-1         | 12.5 ± 4.4 | n.d. | 20.1 ± 12.5 | 14.0 ± 1.9 | 31.0 ± 12.5 | 0 |
| C41G7.3       | 16.3 ± 8.2 | n.d. | 45.5 ± 15.4 | 15.0 ± 2.3 | 13.3 ± 9.2 | 1.4 ± 2.5 |
| pno-1         | 18.8 ± 1.1 | n.d. | 40.2 ± 12.0 | 13.3 ± 1.4 | 14.0 ± 6.8 | 0 |
| mask-1        | 25.1 ± 3.6 | 60.6 ± 5.8 | 50.7 ± 18.6 | 14.7 ± 2.3 | 12.9 ± 3.0 | 0 |

* Pim-6: gfp was scored in L4-Adult worms; n > 160 (range 160–965).
** Top enhancers of Isy-6 activity were scored for uterine cog-1::gfp expression in L4 worms; n > 36 (range 36–106).
*** col-19::gfp was scored in young adult worms; n > 25 (range 25–186).
**** Seam cell number was scored at the same time as col-19::gfp expression in young adults; n > 25 (range 25–186).
***** Synchronized L1 worms were scored at 15°C; vulval bursting scored in day 1 adults; n > 98 (range 98–364).
****** col-19::gfp was scored in young adult worms; n > 18 (range 18–40).
******* n.d. - not determined.
******** Values showing statistical significance are shown in bold.

Figure 2 Several KH domain genes coordinate with Isy-6 to regulate cog-1::gfp expression in uterine cells. (A) RNAi of several KH domain genes, including sfa-1 alleviates the Isy-6-mediated repression of cog-1::gfp in uterine cells (B). ANOVA test was used to determine statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001.

From L4 to adulthood (Reinhart et al. 2000). Compromising let-7 miRNA activity produces a heterochronic phenotype, which, among other defects, includes vulval rupture during the L4-adult molt (Reinhart et al. 2000). let-7(n2853), a temperature sensitive reduction-of-function mutation, causes a mildly penetrant vulval rupture phenotype at 15°C (Figure 5A) (Reinhart et al. 2000). RNAi of six KH domain genes led to significant enhancement of the vulval bursting phenotype (Figure 5B) suggesting these genes may coordinate with let-7 miRNA in a way that normally promotes its activity.

To further explore the genetic interaction between KH domain genes and let-7 miRNA, we asked whether KH domain genes can regulate expression of a let-7 target, lin-41 (Vella et al. 2004). We performed RNAi of four of the genes identified in the let-7(n2853) assay, using three reporter strains. Pdp30::gfp::lin-41 3’UTR
Figure 3 RNAi of multiple KH domain genes enhances the mir-48 mir-241 heterochronic phenotype. (A) Loss of mir-48 mir-241 results in delayed hypodermal expression of the adult specific marker col-19:gfp. (B) When compared to vector RNAi, knockdown of 13 KH domain genes by RNAi enhances the delayed hypodermal col-19:gfp expression of mir-48 mir-241(nDf51) animals. Dots represent experimental replicates. (C) RNAi of some KH domain genes increases the seam cell numbers of mir-48 mir-241(nDf51) young adults when compared to vector RNAi. ANOVA test was used to determine statistical significance. *P < 0.05
also significantly de-repressed GFP::lin-41 (Figure 5, C and D). However, RNAi of the let-7 compromised (Figure 5, C and D). RNAi of the lin-41::gfp expression in hypodermal cells of young adult animals. We used this background to screen for genes that may normally negatively interact with the miRNA pathways and therefore suppress the alg-1(ma202) phenotype when knocked down. Interestingly, RNAi of E02D9.1 significantly suppressed the abnormal hypodermal col-19::gfp expression in alg-1(ma202) young adults, with ~60% of alg-1(ma202) animals showing wild type hypodermal col-19::gfp expression in young adults (Figure 6, A and B). Although not statistically significant, possibly due to the variation in RNAi efficiency, RNAi of other genes (most notably imph-1, sfa-1, akap-1, and Y69A2AR.32) restored wild type col-19::gfp expression in alg-1(ma202) young adults, something that is never observed in alg-1(ma202) mutants alone (Figure 6B). As alg-1 (ma202) suppressors, these KH domain genes may act in a manner that opposes normal miRNA activity, with their depletion perhaps resulting in decreased miRNA target gene expression.

To determine whether any of the KH domain genes might normally have a negative genetic relationship with miRNA pathway components, we performed RNAi knockdown of KH domain genes in the alg-1(ma202) background and assessed col-19::gfp expression in hypodermal cells of young adult animals. We used this background to screen for genes that may normally negatively interact with the miRNA pathways and therefore suppress the alg-1(ma202) phenotype when knocked down. Interestingly, RNAi of E02D9.1 significantly suppressed the abnormal hypodermal col-19::gfp expression in alg-1(ma202) young adults, with ~60% of alg-1(ma202) animals showing wild type hypodermal col-19::gfp expression in young adults (Figure 6, A and B). Although not statistically significant, possibly due to the variation in RNAi efficiency, RNAi of other genes (most notably imph-1, sfa-1, akap-1, and Y69A2AR.32) restored wild type col-19::gfp expression in alg-1(ma202) young adults, something that is never observed in alg-1(ma202) mutants alone (Figure 6B). As alg-1 (ma202) suppressors, these KH domain genes may act in a manner that opposes normal miRNA activity, with their depletion perhaps resulting in decreased miRNA target gene expression.

To determine whether KH domain genes have a general effect on C. elegans development, we assayed the brood size and embryonic lethality of animals with reduced KH domain gene function. Knockdown of seven genes (fubl-4, pes-4, akap-1, E02D9.1, sfa-1, Y69A2AR.32, and bcc-1) resulted in significant reduction in brood size (Figure 7A and Table 2), Depletion of sfa-1 and pes-4 had significant effects on both brood size and embryonic lethality suggesting that these genes play fundamental roles in C. elegans development (Figure 7, A and B, and Table 2). Several additional genes disrupted early development, albeit to a degree that was not statistically significant by our analysis (Figure 7 and Table 2). These observations are consistent with previously reported roles for akap-1, E02D9.1, sfa-1, asd-2, K07H8.9, and bcc-1 in early C. elegans development (Kamath et al. 2003; Sönichsen et al. 2005; Ohno et al. 2008; Ma and Horvitz 2009; Kapelle and Reinke 2011) and highlight additional genes as important for C. elegans fecundity and embryonic development.

Some C. elegans KH domain proteins are evolutionary related and have diverse domain architecture

KH domain containing RBPs play a role in early development

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Some C. elegans KH domain proteins are evolutionary related and have diverse domain architecture

Protein domains are discrete functional and structural segments of a protein. The loss, gain, or structural modification of domains can drive evolution, allowing proteins to lose or acquire new functions over evolutionary time. As domains evolve from ancestral forms, proteins containing the same types of domains may be evolutionary related. To understand the evolutionary relationship between the KH domain-containing proteins and to potentially inform our functional analysis, we performed an alignment of C. elegans KH domain protein sequences using the MEGAx
Figure 5 Several KH domain genes interact genetically with the let-7 miRNA and its target lin-41. (A, B) let-5(n2853) worms display a partially penetrant vulval bursting phenotype at permissive temperature (15 °C). (B) RNAi knockdown of six KH domain genes significantly enhances the vulval bursting phenotype of let-7(n2853) worms. (C) Expression of a reporter system previously designed to assess miRNA activity on miRNA target lin-41 3′ UTR (Ecsedi et al. 2015) in vector control and B0280.17 RNAi. Three strains express Pdpy-30::GFP::lin-41 3′UTR and Pdpy-30::mCherry control in vulval cells: wild type (gfp_lin-41; let-7(+)), let-7(n2853) (gfp_lin-41; let-4(n2853)), and wild type let-7 with lin-41LCS reporter lacking the two functional let-7 complementary sites within the lin-41 3′ UTR (gfp_lin-41LCS; let-7(+)). When let-7 activity is compromised or let-7 sites are removed from lin-41 3′ UTR, GFP expression is de-repressed (quantified in D). (D) RNAi of 4 KH domain genes alleviates the repression on GFP::lin-41 3′UTR expression when let-7 activity is compromised. Images shown in (C) were adjusted post-quantification to allow reader to more easily visualize vulval cells. Cells quantified are highlighted with dashed circles. ANOVA test was used to determine statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
alignment program (Kumar et al. 2018) and generated a phylogenetic tree (Figure 8). Interestingly, proteins that appear to coordinate with miRNAs are found in almost every clade in our phylogenetic analysis (Figure 8).

KH domains are thought to mediate numerous interactions, including those between proteins (Valverde et al. 2008) and proteins and nucleic acids (Grishin 2001; Valverde et al. 2008). Due to the KH domain’s ability to bind RNA, the C. elegans KH domain-containing proteins represent a subset of RBPs, but combinatorial domain arrangements can result in extensive functional diversity among them. To determine the diversity of domain structures of KH domain-containing proteins, we analyzed their domain architecture using the Simple Modular Architecture Research Tool (SMART) (Letunic and Bork 2017), which identifies known domain sequences. In addition, we used the PLACC web-based tool to identify prion-like domains, or unstructured regions (Lancaster et al. 2014). Such low complexity regions are thought to have affinity for RNA (Kato et al. 2012) and can play a role in phase-phase separation that is important for forming and reforming of ribonucleoprotein (RNP) bodies (Shin and Brangwynne 2017). We found that KH domain-containing proteins harbor a diverse set of domains, with prion-like domains present in 17/29 of KH domain proteins (Figure 9). Unsurprisingly, many proteins of the same clade shared additional domains (Figures 8 and 9). These analyses may in the future help inform the mechanisms by which these proteins coordinate with miRNA-mediated regulation of gene expression.

Discussion

KH-domain containing RBPs functionally interact with multiple miRNA families

To determine whether C. elegans KH domain-containing RBPs may function with miRNAs to regulate gene expression, we asked whether RNAi knockdown of KH domain genes could modify the phenotypes observed in reduction-of-function miRNA or family mutants. Surprisingly, eighteen of the twenty-four tested genes genetically interacted with at least one miRNA mutant background, suggesting widespread functional interaction between KH RBPs and miRNAs. Interestingly, the KH domain genes fell into two groups: those that modified phenotypes of all miRNAs sensitized backgrounds tested and those that genetically interacted with specific miRNA reduction-of-function mutants (Table 1). sfa-1 and asd-2 functionally interacted with multiple miRNA families (Table 1), suggesting that these two genes have broad roles in regulation of gene expression. The human orthologs...
of sfa-1, SF1 (Splicing Factor 1), participates in the spliceosome assembly by binding 3' branch sites of pre-mRNAs, whereas its partner, U2AF, cooperatively binds the 5' branch site (Rino et al. 2008). Likewise, the ortholog of asd-2, quaking, has established roles in RNA processing, including alternative splicing and generation of select miRNAs and circular RNAs (Darbelli and Richard 2016). In C. elegans, both sfa-1 and asd-2 are predicted to play a role in splicing, with asd-2 modulating the alternative splicing of unc-60 and other transcripts (Kuroyanagi 2013) and sfa-1 regulating the pre-mRNA splicing of multiple genes (Heintz et al. 2017). Depletion of either sfa-1 or asd-2 was sufficient to induce embryonic lethality and reduce brood sizes (Table 2) (Ma and Horvitz 2017).
consistent with their essential roles as potential global regulators of splicing. Unbiased reverse genetic screens have previously identified splicing machinery members as important for miRNA-mediated gene regulations (Parry et al. 2007). Similarly, factors involved in mRNA processing, including splicing, were found to modulate RNAi efficacy (Kim et al. 2005).

Figure 9 Domain architecture analysis of KH domain containing RNA-binding proteins. Protein domains prediction analysis was performed on the longest predicted isoform using SMART (Letunic and Bork 2017). Region of each protein targeted by RNAi are highlighted below the predicted protein structure. Proteins are grouped in the clades identified via our phylogenetic analysis.
Although splicing and small RNA (including miRNA) pathways intersect, the exact mechanisms by which this occurs remain largely unknown. Given sfa-1 and asd-2 potential roles in splicing, it is perhaps not surprising that these factors show broad genetic interaction with miRNAs across all of our assays.

To better understand the biological context in which miRNAs and KH domain proteins may interact, we compiled spatial KH domain gene expression patterns using existing promoterome (Hunt-Newbury et al. 2007), tissue-specific transcriptome (Kaletsky et al. 2018), and tissue-specific proteome (Reinke et al. 2017) datasets (Supplementary Table S3). Most KH domain genes are broadly expressed with both transcripts and proteins detected in multiple tissues (Supplementary Table S3). For the most part, genes whose RNAi produced a phenotype in a particular miRNA background seemed to be expressed in the relevant tissues. For example, Y69A2AR.52 expression in the hypodermis correlated with its knockdown effects on col-19::gfp expression in mir-48 mir-241 mutant animals (Figure 3). Similarly, most of the KH domain genes whose knockdown resulted in let-6(ot150) phenotype enhancement are neuronally expressed, although it remains unknown whether these KH domain genes are specifically expressed in ASEL/R neurons. Future work is needed to characterize precise tissue and cellular expression to fully understand the spatial and temporal overlap among the molecules in question.

In contrast to the splicing-related factors, the majority of the KH domain-containing RBPs genetically interact with specific miRNAs (Table 1). RNAi knockdown of pes-4 and mask-1 enhances phenotypes of both let-6(ot150) (Figure 1 and Table 1) and mir-48 mir-241 (nDf51) (Figure 3 and Table 1) mutants, suggesting a somewhat general role in gene regulation that spans multiple tissues. By contrast, akap-1, C41G7.3, pno-1, fubl-1, fubl-4, and Y69A2AR.52 genetically interacted with mir-48 mir-241 (nDf51) (Figure 3 and Table 1), but not let-7(n2853) (Figure 5 and Table 1), suggesting a narrower role for these KH domain genes in target gene regulation. Such functional separation can be achieved through differences in temporal expression or perhaps through distinct specificities of RBPs to target miRNAs. In comparison, RNAi of bcc-1, fubl-3 and mxt-1 genetically interacted with both mir-48 mir-241 (nDf51) and let-7(n2853), but not let-6(ot150) (Table 1). The let-7 family shared interactions suggest that these RBPs may have more general roles in developmental timing or may regulate broader sets of target genes. Interestingly, fubl-1 (C12D8.1) was previously identified as a functional interactor of RNAi (Kim et al. 2005), suggesting that this gene’s activity may impact gene regulation carried out by multiple small RNA pathways. tofu-7 was previously identified in a screen for regulators of piRNA biogenesis and function (Goh et al. 2014). In addition, fubl-1, fubl-3, fubl-4, imph-1, and nova-1 show significant phylogenetic clustering with RNAi related genes when integrating existing immunoprecipitation and Drosophila miRNA and siRNA datasets into cluster analysis (Tabach et al. 2013). Taken together, these observations suggest that some of the KH domain genes may coordinate with several small RNA pathways.

**KH domain protein relatedness**

Protein domains are conserved, structured portions of a protein that can fold and function independently. As distinct functional units of a protein, they can dictate, or add to, the overall cellular and molecular role of the protein. Evolution of protein structure and function is in part driven by addition or removal of domains through genetic recombination of domain-encoding gene sequences. To better understand the evolutionary and functional relatedness of the KH domain-containing proteins in *C. elegans* we performed a phylogenetic analysis (Figure 8). Our analysis highlights the overall diversity of these proteins, revealing low levels of similarity between many of the clades, consistent with the observation that in many cases, the proteins sequence similarity is limited to the KH domain(s). However, in contrast to the overall diversity of the proteins, we do see high degrees of relatedness in several of the clades, most notably those containing the FUBL proteins and the grouping consisting of GLD-1 and ASD-2 (Figure 8). This is not surprising given the similarity in domains and overall protein architecture (Figure 9). The phylogeny highlights several clades that genetically interact with miRNAs (Figure 8), perhaps reflecting the functional relatedness relevant to regulation of gene expression. We note that high degree of similarity between the fubl genes could have resulted in some cross-reactivity during RNAi knockdown. However, only RNAi knockdown against fubl-3 could enhance the enhancement of the let-7(n2853) phenotype (Table 1), suggesting some specificity, at least in the case of fubl-3 knock-down.

**Potential models for KH domain RBP and miRNA coordination**

How might KH domain RBPs functionally interact with miRNA pathways to regulate gene expression? Given the evolutionary and domain architecture diversity, the KH RBPs may coordinate with miRNAs, directly or indirectly, via distinct mechanisms. KH RBPs may directly affect aspects of miRNA biogenesis and function or they may indirectly intersect with miRNA pathways by affecting target mRNA processing, transport, stability, and degradation, independent of miRNA activity.

Overall, KH RBPs may exert their gene regulatory effects on miRNA targets indirectly, through multiple effectors (Figure 10A). Alternatively, KH RBPs could more directly regulate the life cycle of miRNA targets by interfacing directly with the miRNAs themselves or with the target mRNAs (Figure 10B). There are multiple mechanisms through which KH RBPs could contribute to miRNA target gene regulation. Proteins involved in splicing, such as SFA-1 and ASD-2, may be involved in splicing events that lead to the production of miRNA transcripts either from their independent gene loci or as part of host mRNA processing (Figure 10C). In this scenario, loss of a splicing factor’s function may reduce the amount of primary miRNA transcript produced, enhancing the reduction of function phenotypes observed in our sensitized background (Figure 10C). In addition, splicing factors may indirectly interact with miRNA pathways by either increasing or decreasing the availability of a gene target (Figure 10D). Alternative splicing of 3’ UTRs that eliminate miRNA target sites has been recently observed (Han et al. 2018). Under this model, KH domain gene depletion could result in alternatively spliced mRNA isoforms that are no longer able to escape miRNA-mediated regulation (Figure 10D), enhancing the phenotypes observed in our reduction of function miRNA mutants.

In another possible scenario, KH domain-containing factors may affect mRNA stability, localization, or transport and thus alter the pool of available miRNA targets (Figure 10E). Increased stability of target mRNAs perhaps through sequestration could reduce miRNA efficacy (Figure 10E). In contrast, reduced stability of miRNA target mRNAs could result in suppression of miRNA-related phenotypes observed in our assays. Interestingly, Drosophila orthologs of MXT-1 (MEXT1) and B0280.17 (HOW) can enhance the stability of mRNAs (Nabel-Rosen et al. 2002; Hernández et al. 2013). The B0280.17 ortholog (HOW) shows isoform dependent enhancement or suppression of mRNA stability.
in order to modulate mRNA translation (Hernández et al. 2013). Likewise, the human orthologs of the FUBL proteins can positively or negatively modulate (depending on the protein) translation of their mRNA targets by binding the 3' UTRs and influencing their stability (Zhang and Chen 2013). These observations lend further support to this model and suggest that the genetic interactions between these RBPs could be complex and context dependent.

Lastly, it is possible that some KH domain-containing RBPs may directly interact with protein components of the miRNA pathway to modulate target gene expression. Several proteins contain additional domains that are predicted to have RNA-binding activity (SAM, zinc finger, splicing factor helix hairpin) (Figure 9) and could mediate interactions among proteins and RNA. Other functional domains such as prion-like or low complexity domains were present in approximately 50% of the RBPs tested. These domains have been implicated in driving liquid phase separation and formation of protein aggregates and RNPs (Putnam et al. 2019). We also see several examples of domains critical for protein-protein interactions, notably the TUDOR domain present in AKAP-1, the STAR homodimerization domains present ASD-2 and GLD-1, and the ankyrin repeats in VGLN-1. Some KH domain-containing RBPs may alter the activity of miRISC by bridging essential protein components or by recruiting additional regulatory factors (Figure 10F). This model is supported by the observation that eight of the twenty-nine KH domain-containing RBPs were previously found to physically interact with miRISC components or DCR-1 (Table 3). MASK-1, FUBL-1, -2, and -3 co-precipitate with AIN-1 (Wu et al. 2017), whereas HRPK-1 and IMPH-1 co-precipitate with DCR-1 (Duchaine et al. 2006) and ALG-1 (Zinovyeva et al. 2015). GLD-1 was found to co-precipitate with ALG-1 (Akay et al. 2013; Zinovyeva et al. 2015) and AIN-2 (Zhang et al. 2007). These proteins may act as scaffolds for the formation of RNP complexes, bridging RNA components (mRNA or miRNA) miRNA biogenesis factors or miRISC (Figure 10F). Overall, KH RBPs could act directly on miRNA targets (Figure 10B) via the suggested mechanisms (Figure 10, A–D) or could indirectly coordinate with miRNAs in regulating gene expression through one or more intermediates (Figure 10A).
Table 3  Several KH domain containing RBPs physically interact with miRISC components

| Gene        | miRISC or biogenesis component |
|-------------|--------------------------------|
| mask-1      | AIN-1<sup>a</sup>             |
| fubl-1      | AIN-1<sup>a</sup>             |
| fubl-2      | AIN-1<sup>a</sup>             |
| fubl-3      | AIN-1<sup>a</sup>             |
| hrpk-1      | ALG-1<sup>b</sup>, DCR-1<sup>c</sup> |
| imph-1      | ALG-1<sup>b</sup>, DCR-1<sup>c</sup> |
| gld-1       | ALG-1, AIN-2<sup>d</sup>      |

<sup>a</sup> Wu et al. (2017).
<sup>b</sup> Zinovyeva et al. (2015).
<sup>c</sup> Duchaine et al. (2006).
<sup>d</sup> Zhang et al. (2007).

Overall, our screen showed that many of the KH domain-containing RBPs in C. elegans functionally interact with miRNA-mediated regulation of gene expression. Further work is essential to characterize the mechanisms through which individual KH domain proteins may affect gene expression and how they might functionally interact with miRNA pathways. This study highlights a number of candidates for future genetic, molecular, and biochemical characterization and shows the extent to which miRNAs and KH domain RBPs may directly or indirectly coordinate to ultimately regulate gene expression.

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