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nhl-2 modulates microRNA activity in *Caenorhabditis elegans*

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Abstract

The *C. elegans* TRIM-NHL protein NHL-2 functions as a co-factor for the *mi*croRNA *I*nduced *S*ilencing *C*omplex (miRISC) and thereby enhances the post-transcriptional repression of several genetically verified microRNA targets, including *hbl-1* and *let-60*/Ras (by the *let-7*-family of microRNAs) and *cog-1* (by the *lsy-6* microRNA). NHL-2 is localized to cytoplasmic processing bodies (P-bodies) and physically associates with the P-body protein CGH-1 and the core miRISC components ALG-1/2 and AIN-1. *nhl-2* and *cgh-1* mutations compromise the repression of microRNA targets in vivo, but do not affect microRNA biogenesis, indicating a role for a NHL-2-CGH-1 complex in the effector phase of miRISC activity. We propose that the NHL-2-CGH-1 complex functions in association with mature miRISC to modulate the efficacy of microRNA:target interactions in response to physiological and developmental signals, and thereby helps ensure the robustness of genetic regulatory pathways regulated by microRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are small RNA molecules that post-transcriptionally repress gene expression by interacting with messenger RNAs (mRNAs). MiRNAs associate with the *miR*NA-*I*nduced *S*ilencing *C*omplex (miRISC), the core of which is composed of a miRNA-specific Argonaute protein (Ago) and its binding partner GW182 (or AIN-1/2 in *C. elegans*) (Eulalio et al., 2008a). This miRNA-*r*ibo*n*ucleo*p*rotein complex (miRNP) recognizes target mRNAs through sequence complementarity between the miRNA and elements in the 3′ *un*t*r*anslated *r*egion (UTR) of the target mRNA. Disruption of the Ago-GW182 complex (by mutation or RNAi-mediated depletion of either component) prevents the miRNA-mediated regulation of target mRNAs (Ding et al., 2005; Eulalio et al., 2008b; Liu et al., 2005; Rehwinkel et al., 2005). Although computational algorithms can identify many potential mRNA targets of a particular miRNA, the precise mechanism(s) and the potency of repression of specific targets likely depends on a variety of determinants. These could include additional cis-regulatory information in the target mRNA sequences and interactions of other proteins with the mRNA or miRISC.

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MiRISC has been shown to control gene expression by affecting at least two aspects of mRNA activity, including the translational efficiency of the mRNA, and/or its stability (for reviews, see (Eulalio et al., 2008a; Hammell, 2008)). Analysis of in vivo miRISC complexes indicates that the repression of miRNA-targeted transcripts involves the physical association of Argonaute proteins with a variety of cellular components, including translational initiation and mRNA turnover machinery (Behm-Ansmant et al., 2006; Chu and Rana, 2006; Eulalio et al., 2007; Mathonnet et al., 2007; Rehwinkel et al., 2005; Wang et al., 2006). Current evidence suggests that miRISC represses protein production from miRNA targets by inhibiting (or competing with) components required for translational initiation, by reducing the efficiency of translational elongation and/or by the miRNA-mediated turnover of target transcripts. However, current models do not adequately account for a) that some miRNA targets appear to be regulated primarily at a translational level while others are regulated by mRNA turnover, or b) that a particular miRNA can have dramatically different potencies on distinct miRNA target reporters (Eulalio et al., 2007). Therefore, it is likely that additional proteins can interact with miRISC to modulate the nature and efficacy of miRISC activity.

Apparent negative modulators of miRISC activity include FXR and HuR, which can interact with miRNA:target complexes and dramatically overcome miRNA-mediated repression (Bhattacharyya et al., 2006; Vasudevan et al., 2007). Knoblich and colleagues have identified members of the *Drosophila* TRIM-NHL and NHL-only families of proteins (Mei-P26, Brat, and Dappled) as miRISC-associated factors, and showed that Mei-P26 can function, directly or indirectly, in the negative regulation of miRNA biogenesis in *Drosophila* ovaries (Neumuller et al., 2008). Mei-P26 phenotypes include elevated levels of miRNAs, and also reduced growth and proliferation of ovarian stem cells (Neumuller et al., 2008). However, it is not known whether the Mei-P26 stem cell phenotypes are a direct consequence of the inappropriate repression of particular miRNA targets.

miRISC-associated factors that positively effect miRNA-mediated target repression include factors that also associate with P-bodies. P-bodies are cytoplasmic domains that contain a variety of important ribonucleoprotein complexes, and can serve as sites of translational repression, decapping-mediated mRNA decay, and nonsense-mediated decay (Parker and Sheth, 2007). Cell culture studies indicate that a key component of P-bodies, the conserved DEAD-box protein RCK/Me31B/Dhh1/CGH-1, are required for miRISC activity (Chu and Rana, 2006; Eulalio et al., 2007). Results from multiple systems indicate that CGH-1 or its homologs can function in various processes of mRNA metabolism, including translational regulation, and mRNA turnover or stabilization (Rajyaguru and Parker, 2008). Genetic analysis indicates that the *C. elegans* CGH-1 homolog functions in the germline to stabilize certain maternal transcripts (Boag, 2008). No miRNA-related phenotypes could be ascribed to CGH-1 in the worm germline, but the question remains whether CGH-1 might contribute to miRISC function during development of the somatic tissues of *C. elegans*.

In this manuscript we show that *C. elegans* NHL-2, the closest worm homolog of *Drosophila mei-P26*, is a miRISC cofactor that functions together with CGH-1 in somatic cells of developing larvae to facilitate the post-transcriptional regulation of certain miRNA-targets. In contrast to the effects of *mei-P26* on miRNA biogenesis in fly ovaries, removal of *C. elegans nhl-2* activity does not affect miRNA abundance, but rather impairs the potency with which certain miRNAs repress their target mRNAs. We show that the P-body component CGH-1 interacts with NHL-2 in yeast 2-hybrid assays, and that CGH-1 and NHL-2 co-immunoprecipitate from worm extracts. Furthermore, both proteins physically and functionally interact with core miRISC components. Depletion of CGH-1 or NHL-2 results in similar post-embryonic developmental phenotypes, suggesting that these proteins work together, likely in association with miRISC, to contribute to the repression of specific miRNA targets. Our results suggest a general role for TRIM-NHL proteins and CGH-1 in modulating the efficacy of

miRNA:target interactions in vivo. These, and perhaps other miRISC cofactors, could function to couple miRNA activity to developmental and physiological signals, and thereby help ensure the robustness of genetic pathways that involve miRNAs.

RESULTS

nhl-2 functions in heterochronic gene regulation

Postembryonic development in *C. elegans* proceeds through four larval stages, accompanied by a robustly controlled temporal and spatial pattern of cell division and differentiation (Sulston and Horvitz, 1977). The heterochronic genes encode components of regulatory pathways that organize the timing of events in specific cell lineages throughout the developing larva. The heterochronic gene *lin-41* encodes a member of an evolutionarily conserved family of TRIM/NHL proteins, which contain both TRIM (Tripartite containing motif; RING, Box, Coiled-Coil) and NHL domains (Slack and Ruvkun, 1998). *lin-41* loss-of-function (lf) mutations cause partially penetrant phenotypes, wherein animals precociously execute adult specific fates one stage earlier than normal (Slack et al., 2000). Since *lin-41(lf)* phenotypes are only partially penetrant, we tested whether *lin-41* may function redundantly with any of the other three members of the *C. elegans* TRIM/NHL gene family (*nhl-1*, *nhl-2* and *nhl-3*). We observed that removal of *nhl-1*, *nhl-2* or *nhl-3* did not enhance the *lin-41* precocious phenotypes, indicating that these genes do not function redundantly with *lin-41*. Quite the contrary; an *nhl-2* loss-of-function mutation suppressed the precocious heterochronic phenotypes caused by a hypomorphic *lin-41* allele, *lin-41(ma104)* (Table 1), suggesting that *nhl-2* contributes to the normal regulation of *C. elegans* developmental timing, but in a manner opposite to *lin-41*. Depletion of the other *C. elegans* NHL family members by RNAi did not result in a similar suppression of *lin-41(ma104)* phenotypes (data not shown).

The *ok818* mutation deletes 1496bp of the *nhl-2* locus, truncating the NHL-2 open reading frame, and is a presumptive null allele (*nhl-2(0)*) (Figure 1 and S1). Approximately three percent of *nhl-2(0)* animals exhibit a retarded heterochronic phenotype; they fail to produce continuous adult specific cuticular structures (alae) at the L4 molt (Table 1), and individual seam cells reiterate L2-specific seam cell lineage patterns at the L3 stage. The relatively weak penetrance of the heterochronic phenotype of *nhl-2(0)* mutants does not reflect a redundancy of NHL-2 with other TRIM/NHL (*nhl-1*, *nhl-3*, *nhl-4*) or NHL-only (*ncl-1*) proteins, as *nhl-3; nhl-2 ncl-1 nhl-1* quadruply-mutant animals display an adult alae phenotype identical to singly-mutant *nhl-2(0)* animals (Table 1).

nhl-2 functions with *let-7*-family miRNAs to control the timing of stage-specific hypodermal cell fates

The low penetrance of the *nhl-2(0)* retarded phenotypes is reminiscent of the defects associated with certain mutants of *let-7*-family miRNA genes. The *let-7*-family miRNAs *mir-48*, *mir-241*, and *mir-84* are required to negatively regulate HBL-1 expression during the L2-L3 transition of larval development by binding complementary elements in the 3′UTR of *hbl-1* transcripts (Abbott et al., 2005). These miRNAs function redundantly; the retarded phenotypes of animals lacking all three *let-7*-family miRNAs are highly penetrant, while the defects of singly-mutant *mir-48(0)*, *mir-241(0)* or *mir-84(0)* animals are weak (Abbott et al., 2005). The retarded phenotypes of animals singly-mutant for individual *let-7*-family miRNAs are dramatically enhanced in combination with an *nhl-2(0)* mutation (Table I). This enhancement is apparent in three independent assays for stage-specific hypodermal cell fates: 1) the delayed spatio-temporal expression of an adult-specific GFP-reporter (*col-19*::GFP), 2) the timing of L2-specific seam cell division programs and 3) the production of adult-specific alae (Figure 1, Table 1 and Table S1). *nhl-2(0)* mutations also enhance the retarded phenotypes associated with a hypomorphic allele of *let-7, let-7(mg279)* (Figure 1E). RNAi depletion of *hbl-1*

completely suppressed the heterochronic defects associated with *nhl-2(0); let-7*-family miRNA compound mutants (Table S1). These results indicate that *nhl-2* functions with or in parallel to the *let-7*-family miRNAs to repress HBL-1 during normal development.

nhl-2 is required for *mir-48* over-expression phenotypes

mir-48(ve33) is a mutation in the *mir-48* promoter that causes over-expression of miR-48, and consequently an abnormally early down-regulation of the miR-48 target *hbl-1*, which results in precocious heterochronic phenotypes (Li et al., 2005). Animals carrying a transgenic array expressing the *mir-48(ve33)* mutant locus (*veIs48*) precociously execute L3 specific vulval divisions at the L2 molt and precociously produce adult alae at the L3 molt (Figure 1F and G). We reasoned that if *nhl-2* promotes the function of *let-7*-family miRNAs, then *nhl-2* could be required for the precocious phenotypes associated with miR-48 over-expression. Consistent with this hypothesis, *nhl-2(0)* suppressed all heterochronic phenotypes associated with *mir-48 (ve33)* (Figure 1F–G). Loss of *nhl-2* did not affect the levels of mature *miR-48, miR-84* or other *let-7*-family miRNAs, suggesting that *nhl-2(0)* suppresses *mir-48(gf)* and *mir-84(gf)* phenotypes by reducing the activity, rather than the amount, of mature *let-7*-family miRNAs (Figure 1H).

nhl-2 functionally interacts with genes encoding core miRISC components

Mutations in *alg-1* (encoding one of the two *C. elegans* miRNA-specific Argonautes) or *ain-1* (encoding one of two *C. elegans* GW182 homologs), result in pleiotropic phenotypes (Ding et al., 2005; Grishok et al., 2001; Zhang et al., 2007), presumably reflecting the roles of *alg-1* and *ain-1* (together with their respective paralogues, *alg-2* and *ain-2*) in the activity of all *C. elegans* miRNAs. The phenotypes of *alg-1* and *ain-1* mutants include developmental timing defects consistent with reduced activity of *let-7*-family miRNAs (Ding et al., 2005; Zhang et al., 2007). To further test the hypothesis that *nhl-2* functions in miRNA pathways, we tested whether *nhl-2* mutations could enhance phenotypes associated with *alg-1(lf)* or *ain-1 (lf)* mutations. *nhl-2(0); alg-1(0)* or *nhl-2(0); ain-1(lf)* animals display strong synthetic heterochronic phenotypes that include defects in adult specific cuticle production (alae) (Figure 1 and Table 1). *nhl-2(0); ain-1(lf)* animals also reiterate L2-specific seam cell proliferation programs (seam cell over-proliferation) at the L3 stage (Figure I). Finally, more than 60% of *nhl-2(0); ain-1(lf)* animals burst from the vulval opening after the L4-molt (n >100), a phenotype characteristic of *let-7*-family miRNA mutants (Abbott et al., 2005). Thus, *nhl-2* loss-of-function strongly enhanced the heterochronic phenotypes of *alg-1* and *ain-1* mutations.

Two lines of evidence suggest that loss of *nhl-2* function enhances the heterochronic phenotypes of *ain-1(lf)* single mutants by reducing the activity of the *let-7*-familiy of miRNAs. First, *nhl-2(0); ain-1(RNAi)* animals fail to properly down regulate the expression of a *hbl-1*::GFP 3′UTR reporter by the end of the third larval stage (Figure 1J). Second, the *col-19*::GFP under-expression phenotypes, adult alae defects, and vulval bursting phenotypes associated with *nhl-2(0); ain-1(lf)* mutations are suppressed by *hbl-1* RNAi (Table S1).

nhl-2 facilitates the negative regulation of the *let-7*-family target *let-60* during vulval development

We hypothesized that *nhl-2* activity could function more broadly with *let-7*-family miRNAs, and play a role in the regulation of *let-7*-family targets other than *hbl-1*. Accordingly, we tested if *nhl-2* activity was required for the proper regulation of another known *let-7*-family miRNA target, *let-60*/RAS (Johnson et al., 2005). *let-60*/RAS plays an essential role in vulval development where it initiates a cascade of gene expression resulting in the specification of vulval tissues (Beitel et al., 1990; Han et al., 1990). Vulva precursor cell fate is sensitive to the dosage of *let-60* activity. *let-60(gf)* alleles result in elevated LET-60 activity and cause ectopic specification of fates in VPCs that would otherwise adopt non-vulval cell fates (Beitel et al.,

1990; Han et al., 1990). For example, *let-60(n1046gf)* is semi-dominant and causes ectopic vulval induction (Muv) in 2% of heterozygous *let-60(n1046)/+* animals (Table S2, and Figure 2). We used *let-60(n1046)/+* as a sensitized genetic background to test for a role for *nhl-2* in the regulation of *let-60* by *miR-84*. *nhl-2(0); let-60(n1046)/+* animals displayed an enhanced Muv phenotype compared to *let-60(n1046)/+* alone (Figure 2B-E and Table S3). A temperature sensitive *let-60(gf)* allele, *let-60(ga89ts)* was also enhanced by an *nhl-2* mutation at the restrictive temperature (Figure 2F and Table S3). Over-expression of *mir-84* has been shown to partially suppress the Muv phenotypes associated with *let-60(gf)* alleles (Figure 2F, Table S3)(Johnson et al., 2005). Consistent with *nhl-2* functioning to promote the activity of *let-7*-family miRNAs, removal of *nhl-2* significantly reduces the suppressive effect of *mir-84* over-expression (Figure 2F and Table S3). Together, these results indicate that *nhl-2* functions in the regulation of at least two *let-7*-family miRNA targets and suggest *nhl-2* may function in miRISC activity.

nhl-2 is required for the efficient post-transcriptional regulation of *cog-1* by the *lsy-6* miRNA

To test the hypothesis that *nhl-2* may function with miRNAs other than *let-7*-family miRNAs, we tested for effects of *nhl-2* mutations on the activity of an unrelated *C. elegans* miRNA, *lsy-6*. *lsy-6* functions in the specification and differential gene expression of two morphologically similar and bilaterally symmetric neurons ASEL and ASER (Johnston and Hobert, 2003). A major determinant distinguishing these two neurons is the ASEL-specific expression of the *lsy-6* miRNA and the resulting down-regulation of a key *lsy-6* target, the transcription factor COG-1 (Johnston and Hobert, 2003). Animals lacking *lsy-6* expression fail to down-regulate COG-1 in ASEL neurons. As a consequence, the ASEL neurons of *lsy-6* null animals (*lsy-6(ot71)*) adopt the ASER fate due to de-repression of COG-1 (Figure 3A and B).

We tested whether *nhl-2* functions in the *lsy-6* pathway by assaying whether *nhl-2(0)* mutations could enhance the weak phenotypes associated with *lsy-6(ot150)*, a hypomorphic allele of *lsy-6*, (corresponding to mutation of a putative ASE promoter element). *lsy-6(ot150)* animals display a partially penetrant ASEL-fate specification phenotype, presumably due to under-accumulation of *lsy-6* miRNA (Sarin et al., 2007)(Figure 3B). The ASEL fate was assayed by scoring for expression of a *lim-6^{pro}::GFP* reporter that is specifically expressed in ASEL and whose expression in this neuron is normally maintained by *lsy-6*. Removal of *nhl-2* activity dramatically enhanced the ASEL fate specification phenotypes associated with *lsy-6(ot150)* (Fig. 3B). These results indicate that NHL-2 functions as a cofactor with *lsy-6* miRNA. Consistent with such a modulatory role, *nhl-2(0)* mutations did not affect ASEL-fate specification in an otherwise wild type genetic background, indicating that NHL-2 is not strictly essential for *lsy-6* miRNA activity (Fig. 3B).

In addition to the role that *cog-1* plays in ASEL/R neuronal specification, *cog-1* also functions in tissues that do not normally express *lsy-6* miRNA, including the vulva and uterus (Palmer et al., 2002). Ectopic expression of *lsy-6* from the *cog-1* promoter results in the down-regulation of COG-1 in vulval and uterine tissues (Johnston and Hobert, 2003). We reasoned that if *nhl-2* functions with *lsy-6* to repress *cog-1* expression, removing *nhl-2* activity should reduce the repression that ectopic *lsy-6* expression exerts on a COG-1::GFP reporter. Consistent with this hypothesis, COG-1::GFP expression was partially restored in vulval, uterine and ASER neurons of animals expressing *cog-1^{pro}::lsy-6* and lacking *nhl-2* (Figure 3C, D and data not shown). Removal of *nhl-2* activity did not affect vulval or uterine expression of COG-1::GFP in animals lacking ectopically expressed *lsy-6*, indicating that *nhl-2* is not involved in *lsy-6*-independent regulation of *cog-1*.

NHL-2 physically interacts with the DEAD-box protein CGH-1 and localizes to cytoplasmic processing bodies

To understand how NHL-2 functions to promote miRNA activity we sought to identify proteins that interact with NHL-2. We performed a yeast 2-hybrid screen with a full-length NHL-2-GAL4(BDB) fusion as bait. Among the clones that strongly interact with NHL-2-GAL4(BDB) were several GAL4-AD-CGH-1 fusions (Figure 4B and C). In parallel experiments, LC-MS/MS analysis of material immunoprecipitated with CGH-1 antisera identified NHL-2 as a candidate polypeptide that co-purified with CGH-1 complexes isolated from *C. elegans* lysates (see supplemental results). The interaction between CGH-1 and NHL-2 was unexpected, because no miRNA-related phenotypes had been associated with *cgh-1* mutations. The presumptively null *cgh-1* mutant (*ok492*) *(cgh-1(0))* develops to adulthood, but is sterile and produces abnormal oocytes and sperm (Boag et al., 2005; Navarro et al., 2001). CGH-1 is expressed primarily in the germ line and early embryo, but is also detectable in somatic P-bodies (Boag et al., 2008).

We investigated whether NHL-2 is also detectable in somatic P-bodies by examining expression of transgenes encoding NHL-2 or CGH-1 fused to fluorescent reporter proteins. In most somatic tissues, CGH-1::GFP was distributed diffusely throughout the cytoplasm, and was also concentrated in punctate cytoplasmic foci that presumably represent P-bodies (Figure 4A and S3). This fluorescence pattern was also evident for GFP::NHL-2 (Figure 4A). Importantly, in animals co-expressing dsRED::NHL-2 and CGH-1::GFP, dsRED and GFP fluorescence was observed in overlapping diffuse and punctate patterns in the cytoplasm of neuronal and hypodermal cells (Figure 4D and data not shown). We addressed the P-body localization of these components further by co-expressing dsRED::NHL-2 with a GFP-tagged translational fusion of DCAP-1, a diagnostic P-body component (Ding et al., 2005). Both proteins were predominantly distributed in coincident cytoplasmic foci (Figure 4E), indicating that NHL-2 likely associates with CGH-1 in P-bodies of somatic cells in vivo.

cgh-1 functions in the heterochronic pathway

If NHL-2 and CGH-1 function together in a complex, one would expect that mutation of either gene would have similar phenotypic consequences. *cgh-1(0)* homozygotes (derived from heterozygous mothers) exhibit mild heterochronic phenotypes that were not detected previously (Table 1 and data not shown). Removal of *cgh-1* function dramatically enhanced the retarded heterochronic phenotypes associated with *mir-48* and *mir-84* loss of function mutations. As was observed for *nhl-2(0); let-7*-family compound mutants, hypodermal seam cell lineages of *cgh-1(0); mir-48(0); mir-84(0)* animals exhibit enhanced heterochronic developmental defects, including reiteration of L2-specific proliferative divisions during the L3 stage, and defects in adult-specific gene expression (*col-19::GFP*) (Table 1, Figure 5A–C). These heterochronic defects also cause *cgh-1(0); mir-48(0); mir-84(0)* animals to burst from the vulva at the L4/Adult transition, a phenotype associated with reduced *let-7*-family activity. *cgh-1(0)* mutations strongly enhance heterochronic phenotypes associated with reduced activities of the core miRNA machinery (Table 1). The seam cell lineage defects, adult alae, vulval bursting and *col-19*::GFP under-expression phenotypes displayed by *cgh-1(0); mir-48(0); mir-84(0)* animals are suppressed by *hbl-1* RNAi (Figure 5C and Table S2), indicating that CGH-1 is required, along with *let*-7-family miRNAs, for the proper down regulation of HBL-1.

nhl-2 and *cgh-1* function in concert to regulate the *let-7*-family miRNA target hbl-1

The above results, showing that either *nhl-2(0)* mutations or *cgh-1(0)* mutations can sensitize animals to reduction of *let-7*-family miRNA function (Figure 1C–E, 5A–C), indicate that NHL-2 and CGH-1 may function in concert to promote *let-7*-family activity. Consistent with this conclusion, we found that depletion of *cgh-1* activity in *nhl-2(0)* mutant animals results in

retarded heterochronic phenotypes far more severe than *nhl-2(0)* or *cgh-1(0)* alone, and resulted in a perdurance of *hbl-1::GFP* 3′UTR reporter in L3-staged animals (Figure 5D-G and I). In addition to the enhanced heterochronic phenotypes associated with *cgh-1(0) nhl-2(0)* double mutants, greater than 40% of *cgh-1(0) nhl-2(0)* animals (derived from heterozygous mothers) die during embryogenesis or early larval development, indicating that the combined activities of *nhl-2* and *cgh-1* are required for viability (Figure 5H). This suggests that NHL-2 and CGH-1 may act together to influence the activity of additional miRNAs besides *lsy-6* and *let-7* family miRNAs.

NHL-2 and CGH-1 associate with the miRISC complex

Depletion of CGH-1 (via RNAi) does not appear to affect the biogenesis of miRNAs, nor does mutation of *nhl-2*, (Figure 1H and 6A) suggesting that CGH-1 and NHL-2 function in step(s) after miRNA biogenesis. The yeast 2-hybrid interaction between CGH-1 and NHL-2, their co-localization in P-bodies, and the genetic interactions among *nhl-2, cgh-1, let-7*-family miRNAs, and *lsy-6* miRNA, suggest that the CGH-1-NHL-2 complex may associate with components of the *C. elegans* miRISC. Consistent with this supposition, immunoprecipitation of the GFP::NHL-2 using anti-GFP antibodies co-precipitated components of core-miRISC machinery (Figure 6B). In addition, western blot analysis of complexes co-immunoprecipitated with CGH-1 indicated that CGH-1 interacts in vivo with NHL-2, and with the core-miRISC components ALG-1, ALG-2 and AIN-1 (Figure 6C). The fraction ALG-1/2 and AIN-1 co-precipitated with GFP::NHL-2 or CGH-1 immunoprecipitation was relatively low, perhaps reflecting instability of these interactions in extracts. The physical association of CGH-1 with the core-miRISC components is preserved in *nhl-2(0)* mutant animals, indicating that binding of CGH-1 to miRISC is not mediated by NHL-2 (Figure 6D).

Interestingly, RNAse treatment of immunoprecipitated complexes dramatically reduced the association of AIN-1 and AGL-1/2 with CGH-1, but did not dissociate NHL-2 from CGH-1 (Figure 6C). These result are consistent with the strong interaction of CGH-1 and NHL-2 detected by yeast 2-hybrid assays, and furthermore suggest that in vivo CGH-1 and NHL-2 engage in a relatively strong, mutual interaction, while the binding of the CGH-1: NHL-2 complex to miRISC is more indirect, and depends in part on RNA-protein interactions.

Discussion

nhl-2 modulates miRNA-mediated regulation of cell fate specification

In this manuscript we identify roles for *nhl-2* in the miRNA-mediated posttranscriptional regulation of three key developmental control genes. The first case involves the stage-specific down-regulation of the transcription factor HBL-1, which must occur properly to trigger developmental switches in cell fate that normally occur between the second and third larval stages. This down-regulation of HBL-1 requires the efficient activity of the *let-7*-family of miRNAs, and we show here that NHL-2 contributes positively to *let-7*-family repression of HBL-1. Secondly, in *C. elegans* vulval development, *let-7*-family miRNAs contribute to the negative regulation of *let-60*/RAS, a critical signaling component involved in specifying certain vulval cell fate choices. We show that NHL-2 promotes *let-7*-family activity in this spatial patterning of vulval cell fates. The third case involves the *lsy-6* miRNA, which is required in certain neurons to confer left/right asymmetric expression of the transcription factor COG-1, and thereby to specify particular neuronal cell fates. We show that NHL-2 functions to promote *lsy-6*-mediated repression of COG-1 in neuronal cell fate specification, and also in other cells when we expressed *lsy-6* ectopically. These results demonstrate that *C. elegans* NHL-2 protein is required in diverse developmental contexts to promote the robustness of cell fate choices mediated by miRNA:target interactions.

NHL-2 functions as a co-factor for miRNA activity

Our results also demonstrate that *C. elegans* NHL-2 (and by implication, other TRIM-NHL family homologs) can function directly in the effector phase of miRNA-mediated post-transcriptional regulation. In support of a direct role for NHL-2 in miRISC activity are our findings that NHL-2 i) interacts in vivo with miRISC, ii) is genetically required for the full potency of *let-7*-family and *lsy-6* miRNA activities, but iii) does not detectably affect overall miRNA levels (Figure 6). We propose that *nhl-2* functions as a modulatory co-factor for miRNA activity, based on our observations that i) *nhl-2* function is not essential for miRNA activity, yet ii) *nhl-2* mutations enhance the loss-of-function phenotypes of particular miRNA genes (Figure 1, 3 and Table 1) and iii) *nhl-2* mutations suppress the gain-of-function phenotypes associated with over-expression or ectopic expression of miRNAs (Figure 1–3).

Our finding that the TRIM-NHL protein NHL-2 functions positively to promote miRNA activity in *C. elegans*, without apparent effects on miRNA biogenesis, represents an interesting counterpoint to recent findings regarding *mei-P26*, the closest *Drosophila* homolog of *nhl-2*. Mutations of *mei-P26* result in a variety of phenotypes that include over-proliferation of undifferentiated germ line tissues and alterations in neuronal development (Glasscock et al., 2005; Neumuller et al., 2008). Molecular characterization of *mei-P26* mutants indicates that, in the germ line, Mei-P26 functions to repress the biogenesis of numerous miRNAs (Neumuller et al., 2008). Neumuller et al. also report that (similar to our findings for NHL-2), Mei-P26 physically associates with the miRISC complex. Although the apparent conservation of a general TRIM-NHL-miRISC interaction is striking, in the case of Mei-P26, an indirect role in miRNA accumulation was not ruled out. Moreover, it was not possible to link the *mei-P26* mutant phenotypes to the derepression or over-repression of particular miRNA targets, so those developmental defects could reflect miRNA-independent functions for Mei-P26. The apparent differences in the modes of activity of NHL-2 and Mei-P26 could also reflect different roles for these proteins in germ-line vs. somatic tissues. In any case, our data show definitively that in somatic cells, TRIM-NHL/miRISC interactions positively modulate miRISC activity.

A key finding from our experiments is that NHL-2 interacts physically and functionally with the P-body-associated DEAD box protein CGH-1. We observe that *C. elegans* NHL-2 and CGH-1 are broadly expressed in diverse cell types (both germ-line and somatic tissues), and hence may function in a variety of developmental processes. We show that in somatic cells, NHL-2 and CGH-1 are distributed throughout the cytoplasm and also co-localize in cytoplasmic foci. These foci correspond to P-bodies, which have been implicated in translational repression and decapping-associated mRNA turnover (Parker and Sheth, 2007). The localization of NHL-2 and CGH-1 to P-bodies and the somatic phenotypes associated with *nhl-2(0)* and *cgh-1(0)* mutations suggest that miRNA-mediated mRNA turnover and/or translational repression may be facilitated by P-bodies. While the roles of P-bodies in miRNA-mediated repression are not yet fully understood, several structural and enzymatic components of P-bodies have also been shown to contribute to miRNA-mediated regulation (including GW182 and the decapping and mRNA turnover machinery)(Behm-Ansmant et al., 2006; Eulalio et al., 2008b; Eulalio et al., 2007; Liu et al., 2005; Rehwinkel et al., 2005).

Our finding that *C. elegans* CGH-1 functions to regulate HBL-1 expression somatic cells is consistent with a conserved role for CGH-1 (and CGH-1 homologs) in miRNA-mediated gene regulation (Chu and Rana, 2006). Previous biochemical and genetic analyses of *C. elegans* CGH-1 indicate that CGH-1 is localized to germ line P-granules (germ line-specific mRNA storage compartments) and functions in the germ line to sequester and stabilize certain maternally transcribed mRNAs, possibly in association with a highly conserved family of RNA-binding proteins, (Boag et al., 2008; Boag et al., 2005). It is thought that, in the context of the germ line CGH-1's activity and localization to distinct mRNA storage bodies is modulated by additional factors (including germ line-specific RNP-components)(Rajyaguru

and Parker, 2008). A role for CGH-1 in mRNA stabilization of germ-line mRNAs is clearly distinct from the well-characterized role the *Drosophila* CGH-1 homolog (Me31B) plays in the degradation of somatic mRNAs (Eulalio et al., 2007). Homologs of CGH-1 (Dhh1p in *S. cerevisiae* and RCK/54 in humans) have also been demonstrated to promote mRNA decapping and/or translational repression (Coller and Parker, 2005; Coller et al., 2001; Eulalio et al., 2007).

Our findings are consistent with numerous possible molecular models for NHL-2 activity in miRNA-mediated regulation. The essence of these models would be that NHL-2 function involves its association with miRISC, and in particular, direct binding to CGH-1. Moreover, the apparent RNAse-sensitivity of the interaction of the CGH-1:NHL-2 complex with miRISC indicate that CGH-1 and NHL-2 function in association with mRNA targets. The NHL-2 protein contains multiple domains (Figure 1B), including B-box domains, which have been implicated in RNA binding and protein-protein interactions (Hall, 2005; Matthews and Sunde, 2002). So, in principle, NHL-2 could interact with miRISC and target mRNAs by RNA-binding and/or protein-protein interactions.

Models for NHL-2 activity can be broadly classified according to how NHL-2 may facilitate interactions between miRISC and miRNA targets or modulate repressive activities mediated by miRISC components. The first class of models would posit that NHL-2 associates with target mRNPs and helps stabilize the association of CGH-1-miRISC with target mRNAs and/or promote the fidelity of target recognition (Figure 6E). Our finding that CGH-1 remains associated with the protein components of miRISC in *nhl-2(0)* mutants, indicates that NHL-2 does not affect CGH-1 binding to miRISC (Figure 6D), but does not rule out the possibility that NHL-2 could facilitate binding to targets of a miRISC-CGH-1 complex. For technical reasons, we could not examine the association of NHL-2 to miRISC in a CGH-1 null, so we cannot say whether NHL-2 binds to miRISC via CGH-1, or independently of CGH-1. Therefore, strictly speaking, our data do not distinguish whether CGH-1 and NHL-2 function interdependently, or whether each exerts distinct and independent effects on miRNA-mediated gene regulation.

In a second class of models, NHL-2 may associate with previously formed miRISC:target mRNA complexes and stimulate the repressive activity of miRISC (Figure 6E, 2). In this scenario, NHL-2 would bind miRISC and stimulate the intrinsic repressive activity of one or more miRISC components (such as CGH-1) (Figure 6E, 2). CGH-1 is a member of the DEAD-box family of RNA-dependant ATPases that play a variety of roles in RNA metabolism including the rearrangement of protein-protein, protein-RNA, and RNA-RNA complexes. Therefore, NHL-2 could affect multiple activities of CGH-1, or could preferentially stimulate translational inhibitory or mRNA degradative functions of CGH-1. NHL-2 could act independently of CGH-1 and simply use its physical association with miRISC to exert NHL-2-specific activities to target mRNAs, their nascent protein products, or other cellular components involved in miRNA-mediated repression (Figure 6E). NHL-2 and other TRIM-NHL family proteins contain RING-domains, which have been implicated in the post-translational ubiquitination of target proteins. Ubiquitination serves a variety of functions that include protein turnover, modulation, and differential subcellular localization of target proteins. In this role (Figure 6E, 3), NHL-2 may directly modify specific components of the mRNA turnover or miRISC machinery, which would in turn modulate their activity on miRNA targets. Preliminary results indicate that neither CGH-1 nor core miRISC components (ALG-1/2 and AIN-1) are significantly poly-ubiqutinated in vivo and the absence of NHL-2 does not lead to the mislocalization of CGH-1, ALG-1/2, or AIN-1 from somatic P-bodies (Figure S4 and data not shown). Alternatively, NHL-2 could function as a miRISC-associated ubiquitin ligase that modifies nascent polypeptides encoded by miRNA-targets or ribosomes engaged with miRISC (Figure 6E, 3).

We show that NHL-2 functions as a cofactor for *let-7*-family miRNAs, and also for an unrelated miRNA, *lsy-6*. This may not represent the full range of miRISC-associated NHL-2 specificity, as *nhl-2 cgh-1* doubly-mutant animals display defects in somatic cell development and viability not known to be associated with reduction in *let-7*-family or *lsy-6* miRNAs. However, our results to date do not indicate that NHL-2 functions in conjunction with all miRNAs in *C. elegans*, as we have not observed genetic interactions between *nhl-2(0)* and miRNA gene mutants other than the *let-7*-family and *lsy-6*. In sum, we propose that the modulation of miRISC activity by NHL-2 (and potentially other TRIM-NHL proteins) functions to sharpen the post-transcriptional regulation of critical miRNA targets and may modulate miRNA efficacy in response to physiological or developmental signals, and hence ensure the robustness of genetic regulatory pathways involving miRNAs.

MATERIALS AND METHODS

Nematode Methods

C. elegans strains were grown under standard conditions (Brenner, 1974). Transformation of animals and RNAi were performed as previously described (Abbott et al., 2005; Mello et al., 1991).

Quantification of miRNA Expression

Total RNA was isolated from either staged or mixed populations of worms and Northern blots were performed as previously described (Lee and Ambros, 2001).

Microscopy and Phenotypic Analysis

Lineage analysis and scoring of adult alae phenotypes were performed by picking staged animals of the indicated genotypes and monitoring seam cells derived from the V lineage as described by Sulston and Horvitz (Sulston and Horvitz, 1977).

2-hybrid screening

NHL-2 interacting proteins were identified using Clonetec Matchmaker according to the manufacturer's protocol. Interactions between NHL-2 and potential interacting proteins were assayed in the PJ69-4a yeast cells. GAL4-AD fusion libraries were a gift of R. Barsted.

Protein Analysis

A detailed description of lysate preparation, immunoprecipitation and antibody protocols can be found in supplemental text. A detailed description of the LC-MS/MS analysis of CGH-1 interacting proteins can be found in supplemental materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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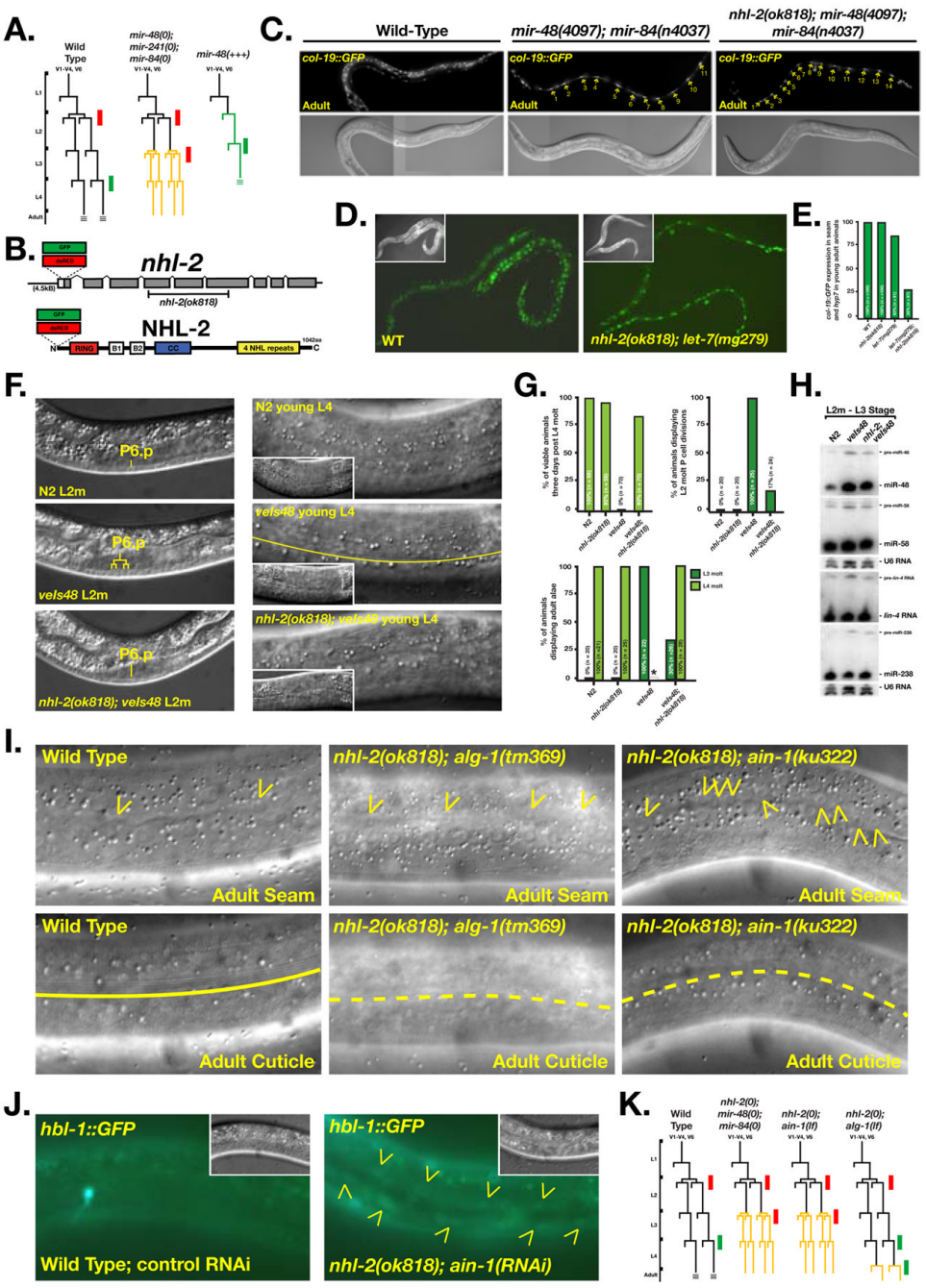

Figure 1. *nhl-2* functions with the *let-7*-family of miRNAs and core miRISC components to regulate heterochronic gene expression

(A) The lateral seam cells (V1-4 and V6) of wild type animals execute stage-specific division patterns. At the L1, L3 and L4 stages, each seam cell executes a single asymmetric division, producing an anterior cell that fuses with the hyp7 syncitium, and a posterior daughter seam cell. At the L2 stage, a different pattern occurs: each seam cell divides symmetrically, and each daughter seam cell immediately divides again asymmetrically (red bars). This L2-specific duplicative seam cell division essentially doubles the number of seam cells in the hypodermis of *C. elegans*. In animals triply mutant for *mir-48, mir-241*, and *mir-84*, a reiteration of L2-specific seam cell division patterns occurs at the L3 stage. In contrast, animals over-expressing

of miR-48 skip most L2 seam cell division programs, precociously execute vulval divisions and the produce adult specific cuticular structures at the end of the L3 stage. **(B)** *nhl-2* encodes a member of the TRIM-NHL family of proteins and the *ok818* deletion is a null allele of *nhl-2* (Fig. S1). **(C)** In wild-type animals, *col-19::*GFP expression is adult-specific, and occurs in lateral seam and in hyp7 cells. In doubly-mutant *mir-48; mir-84* adults, *col-19::GFP* expression is reduced in hyp7, and limited primarily to seam cells (arrows). In *nhl-2; mir-48; mir-84* triply-mutant adults, hyp-7 *col-19::*GFP expression is reduced further, and other heterochronic phenotypes occur, including a reiteration of L2-specific seam cell division patterns in some V-lineages. **(D and E)** *nhl-2* mutations similarly enhance the *col-19::*GFP expression phenotypes of *let-7* mutants. **(F and G)** The precocious vulval induction, seam cell fusion and adult alae formation phenotypes associated with miR-48 over-expression are suppressed by removing *nhl-2*. Asterisks indicate that lateral seam cells of *veIs48* animals precociously exit the cell cycle after the L3 molt. **(H)** Northern analysis of miRNAs derived from L2 molt wild-type, *veIs48,* and *nhl-2(0);veIs48* animals. U6 RNA indicates loading controls. **(I)** *nhl-2(0)* enhances the L2-specific seam cell phenotypes associated with *ain-1 (ku322)* mutations and also enhances L4-to-adult transition defects in *alg-1(tm369)* animals. Arrows indicate seam cell nuclei, solid lines indicate areas of cuticle containing adult-specific alae, and dashed lines indicate areas of cuticle lacking alae. **(J)** RNAi depletion of *ain-1* in *nhl-2(0)* animals prevents the posttranscriptional down regulation of *hbl-1::*GFP. Arrows indicate the nuclear expression of the *hbl-1::*GFP reporter in hyp7 cells of L3 *nhl-2(0); ain-1 (RNAi)* animals. **(K)** Proposed lineage diagrams that illustrate the synthetic phenotypes associated with *nhl-2(0)* and mutations of *let-7*-family miRNAs or of core miRISC components. Red bars indicate L2 specific cell division programs and green bars indicate those associated with L3 molt-L4 seam divisions.

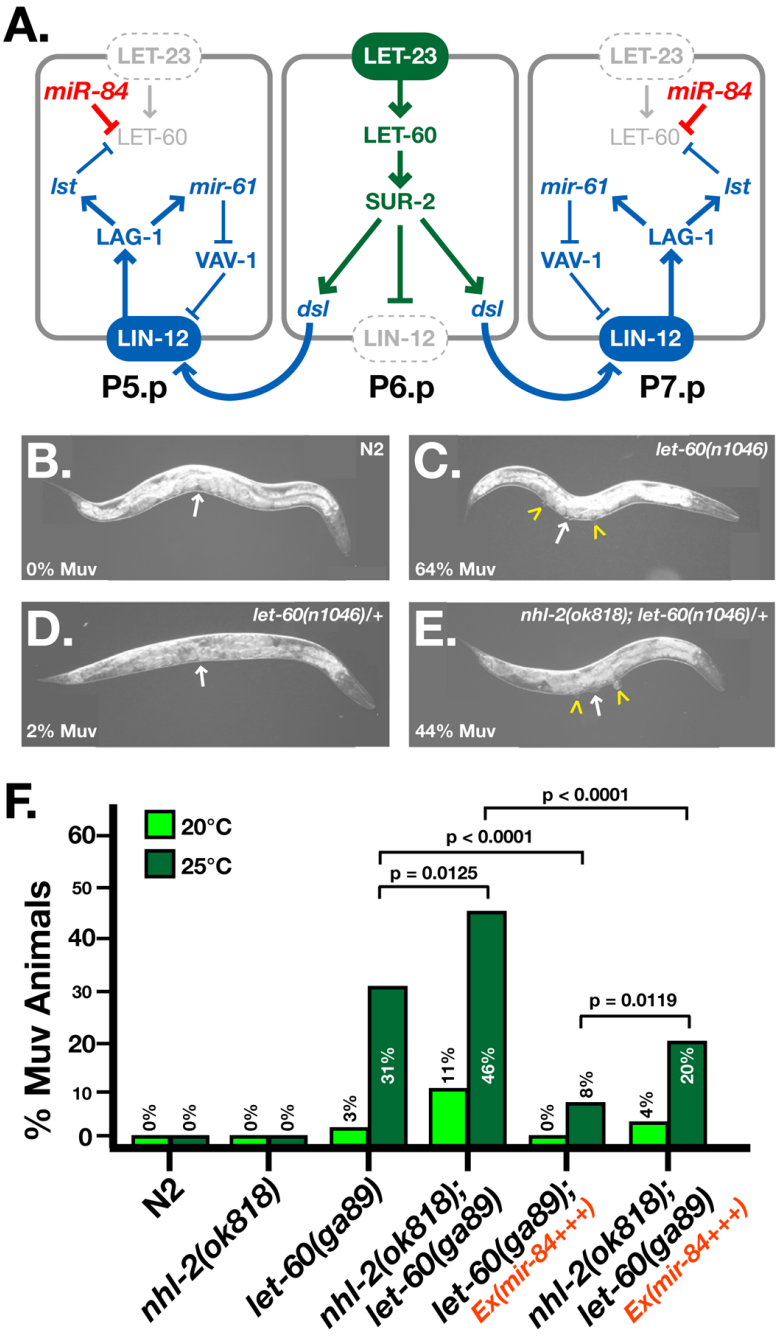

Figure 2. *nhl-2* negatively regulates *let-60* activity

(A) The major components of the vulval induction pathway (See text for details). Components that are genetically involved in vulval induction are colored green where as those involved in Notch/lateral inhibition are colored blue. The *let-7*-family miRNA miR-84 (Red) is temporally expressed in presumptive 2° vulval cells. **(B–E)** *nhl-2(0)* mutations enhance *let-60(n1046gf)*, Muv phenotypes. **(F)** *nhl-2* mutations enhance *let-60(ga89)* temperature sensitive Muv phenotypes. Over expression of miR-84 suppresses the Muv phenotypes of *let-60(ga89)*. The suppression of *let-60(ga89)* Muv phenotypes by miR-84 is reduced in animals lacking *nhl-2*. Brackets indicate statistically significant differences in phenotype calculated from a two-tailed chi-squared analysis from data derived from Table S3.

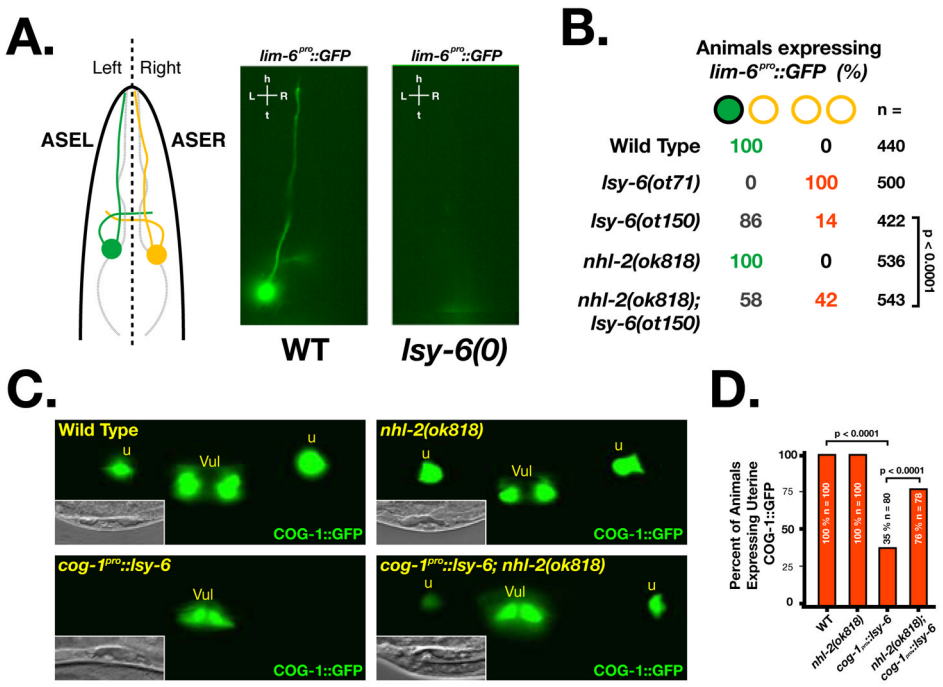

Figure 3. ***nhl-2*** **function is required for the efficient regulation of** ***cog-1*** **mRNAs via the** ***lsy-6*** **miRNA**
(A) A diagram of *C. elegans* larva illustrating the location of ASEL and ASER neurons. *lsy-6* mutants result in an absence of *lim-6*^{pro}::GFP expression in ASEL neurons. **(B)** Quantification of *lim-6*^{pro}::GFP mis-expression phenotypes in *lsy-6*, *nhl-2* and *nhl-2; lsy-6* compound mutants. **(C)** Ectopic expression of the *lsy-6* miRNA from the *cog-1* promoter results in the inappropriate down-regulation of COG-1::GFP in vulval and uterine tissues. Removing *nhl-2* in the context of ectopic *lsy-6* expression restores COG-1::GFP expression in uterus (Vul=vulva and u=uterus). **(D)** Quantification of uterine COG-1::GFP expression in various mutants. Brackets in B and D indicate statistically significant differences calculated from a two-tailed chi-squared analysis.

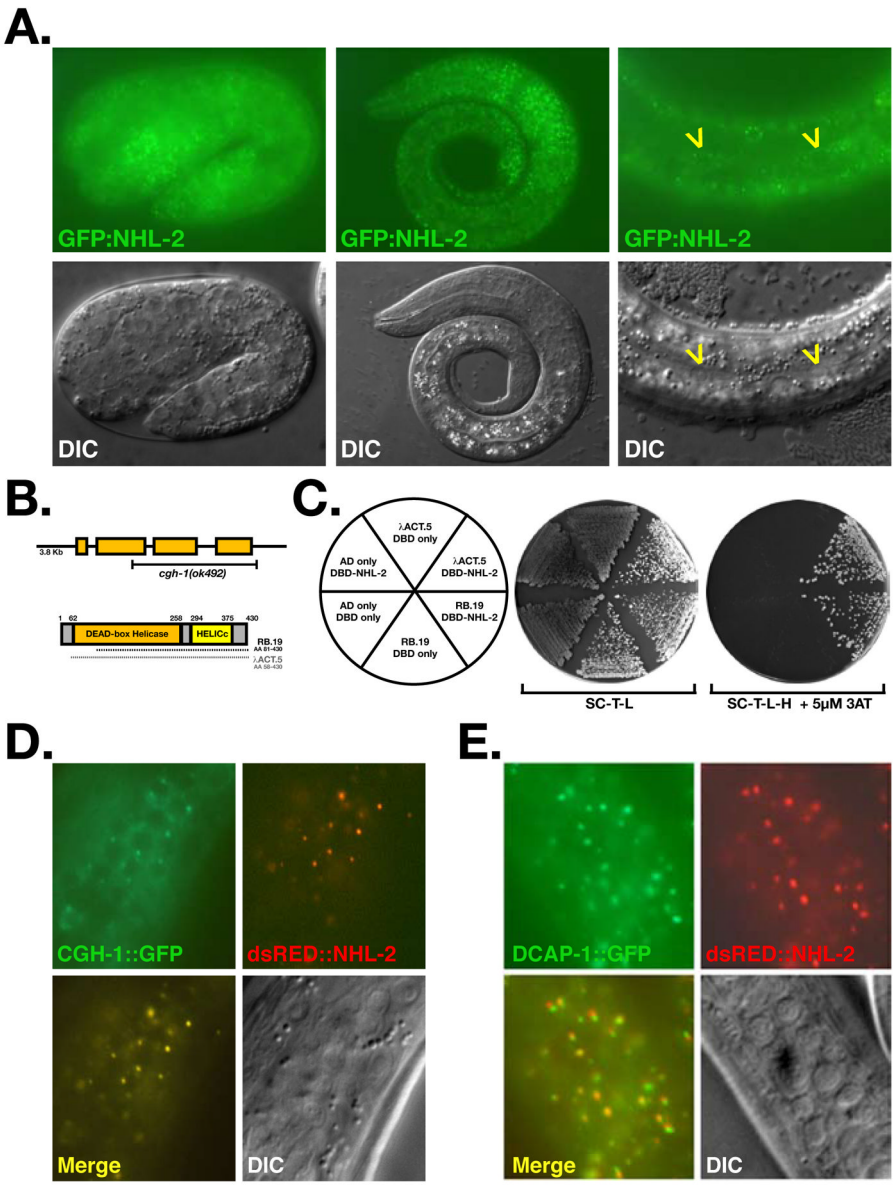

Figure 4. NHL-2 is a cytoplasmic protein that co-localizes with somatic P-bodies and physically interacts with the conserved DEAD-box protein CGH-1

(A) A GFP::NHL-2 transgene is expressed in a variety of embryonic and larval tissues throughout development**. (B)** The *cgh-1* genomic locus, with the location of the *ok492* mutation indicated, and CGH-1 protein domain configuration, with ORF sequences identified in the NHL-2 2-hybrid screen (dashed lines). **(C)** Growth phenotypes of yeast strains co-transformed with various bait and prey constructs on non-selective or selective medium. **(D and E)** CGH-1::GFP and dsRED::NHL-2 co-localize in somatic tissues to several punctate, cytoplasmic bodies. dsRED::NHL-2 also co-localizes a translational DCAP-1::GFP transgene encoding the resident somatic P-body component.

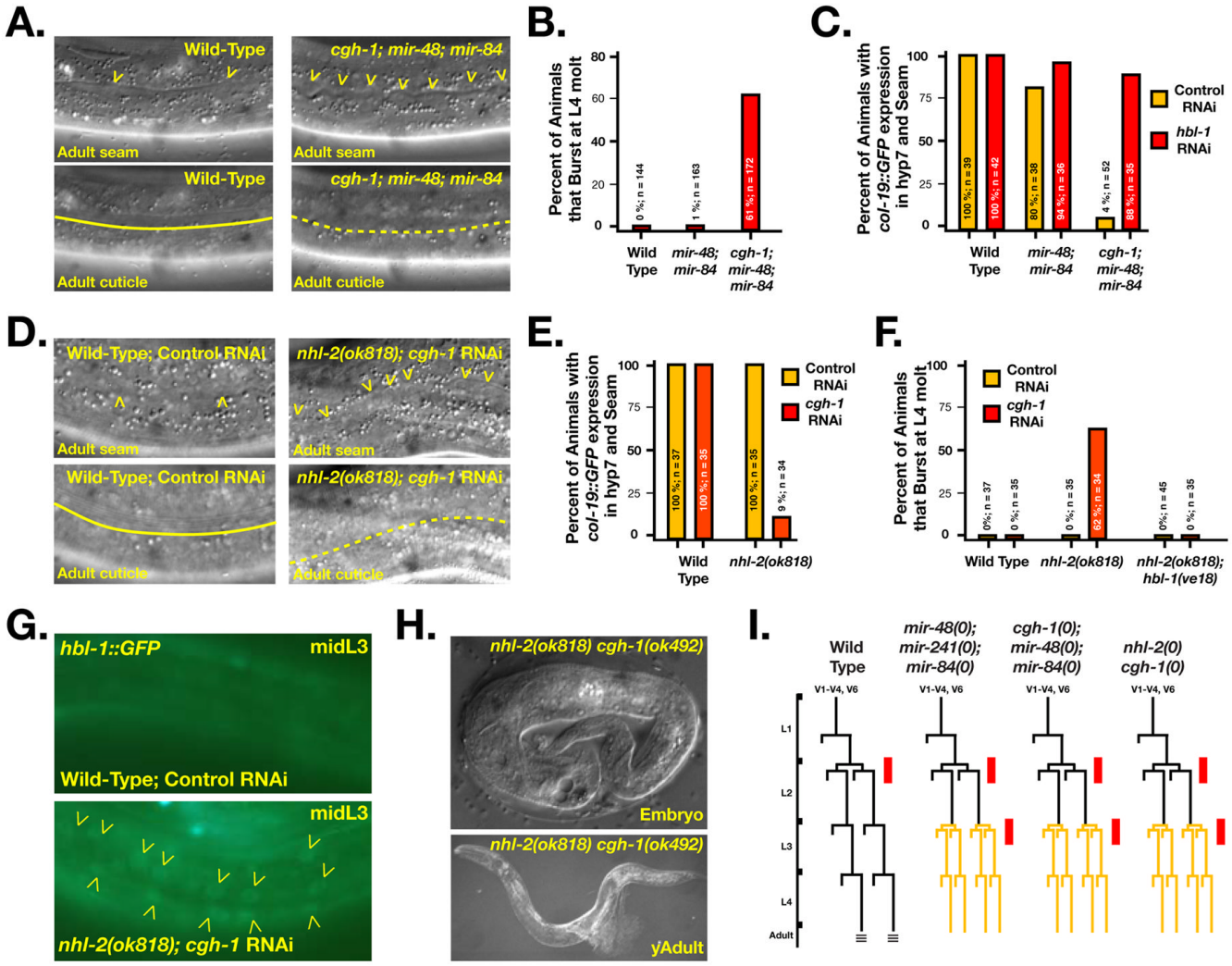

Figure 5. NHL-2 and CGH-1 are essential for the *let-7*-mediated down-regulation of HBL-1 expression

(A) *cgh-1(ok492); mir-48(n4097);mir-84(n4037)* lack adult alae (yellow line in wild type panel (left)) and contain supernumerary seam cells (arrowheads). **(B)** *cgh-1(ok492); mir-48 (n4097);mir-84(n4037)* animals display a highly penetrant, vulval bursting phenotype that is not displayed in *cgh-1(ok492)* or *mir-48(n4097); mir-84(n4037)* mutants. **(C)** Animals were hatched onto *hbl-1* RNAi food and assayed for adult specific *col-19::GFP* expression in early adulthood. **(D)** RNAi of *cgh-1* in *nhl-2(0)* mutant backgrounds results a reiteration of L2-specific seam cell division patterns and adult *nhl-2(0); cgh-1* RNAi animals display supernumerary seam cells and lack alae. **(E and F)** *nhl-2(0); cgh-1* RNAi animals display defects in the expression of the adult-specific *col-19::GFP* transcriptional reporter and burst at the L4 molt. Mutations of *hbl-1* dramatically reduce the penetrance of heterochronic phenotypes associated with *cgh-1* RNAi in *nhl-2(0)* animals including vulval bursting. **(G)** The *hbl-1* 3′UTR GFP reporter is mis-expressed in *nhl-2(0); cgh-1(RNAi)* animals. **(H)** *cgh-1(0) nhl-2(0)* mutants also display a synthetic lethal phenotype during embryogenesis and larval development. **(I)** Lineage diagrams illustrating defects in temporal seam cell division programs of *let-7*-family miRNA mutants, compound *cgh-1(0); let-7*-family miRNA mutants, and *nhl-2 (0) cgh-1(0)* mutants. Red bars indicate L2 specific cell division programs.

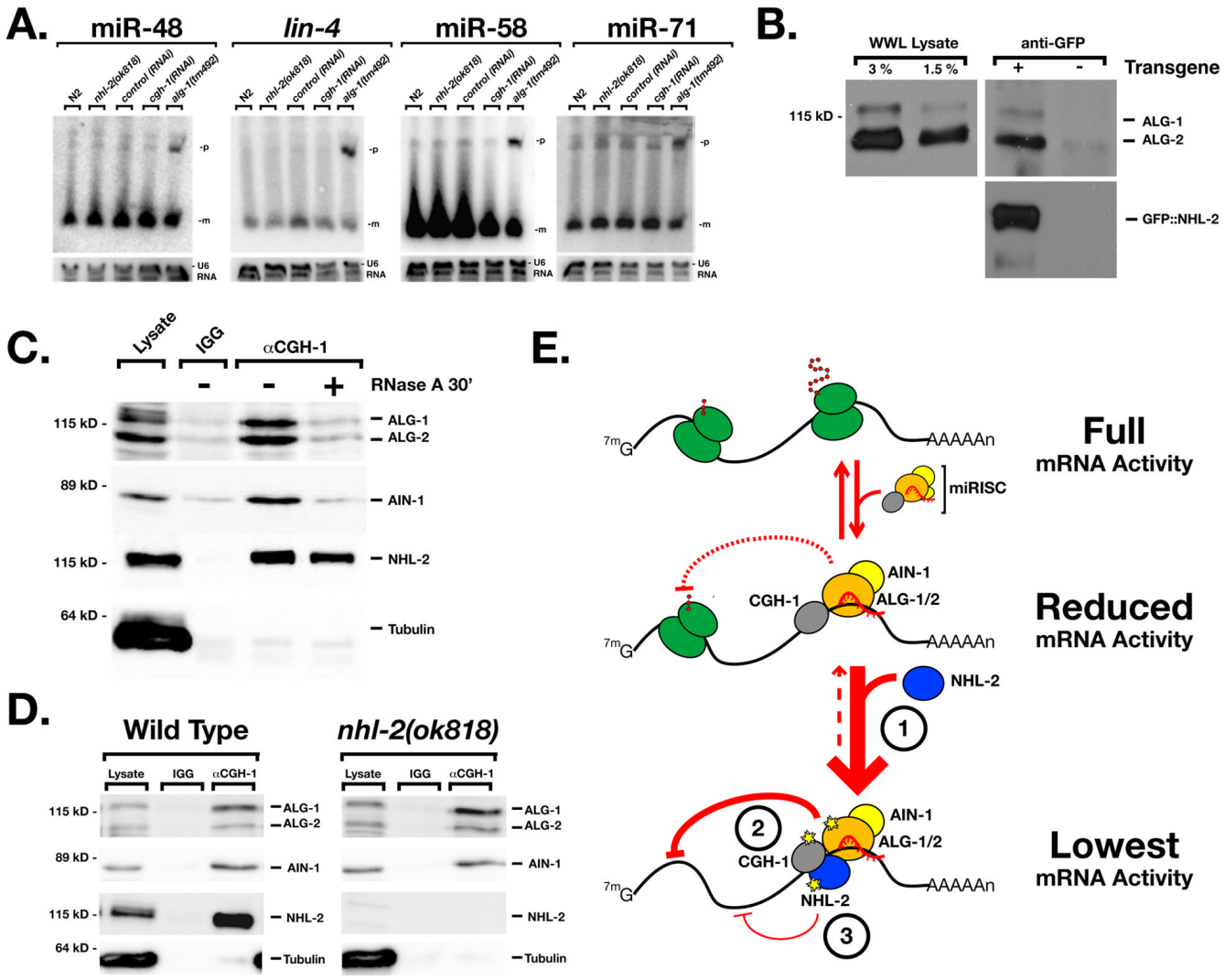

Figure 6. NHL-2 and CGH-1 act after miRNA biogenesis to promote miRNA-mediated gene regulation in association with the core miRISC

(A) miRNA Northern analysis of total RNA extracted from wild-type, *nhl-2(0)*, negative control RNAi, *cgh-1* RNAi or an *alg-1(0)* mutant. Only mutations of *alg-1*, encoding the miRNA-associated Argonaute and core miRISC component, lead to defects in miRNA biogenesis (miss-processing and under-accumulation of mature miRNAs). **(B)** Immunoprecipitation of GFP::NHL-2 from extracts larvae co-precipitates ALG-1 and ALG-2. **(C)** Immunoprecipitation of CGH-1 co-precipitates ALG-1/2, AIN-1, and NHL-2. RNAse treatment of immunoprecipitated material reduces CGH-1 binding to core miRISC components but does not dramatically effect its interaction with NHL-2. **(D)** Co-immunoprecipitation of ALG-1/2 and AIN-1 by CGH-1 antiserum is not affected in the absence of *nhl-2*. **(E)** A model representing potential roles for NHL-2 in the post-transcriptional regulation of miRNA targets. We suggest that miRISC has a basal level of activity in the absence of NHL-2 and can lead to the reduction of protein products derived from miRNA target transcripts. Our models suggest that NHL-2 augments this basal level of activity by physically associating with miRISC. Proposed points of function and potential activities NHL-2 include 1) an enhancement of CGH-1-miRISC-target interaction by increasing the stability or fidelity of miRNA:target interactions; 2) a stimulatory role for NHL-2 in modulating intrinsic activity of CGH-1 and/or

miRISC components; and the 3) a role for NHL-2 independent of CGH-1 or other known miRISC components.

Table 1

Analysis of genetic interactions of *nhl-2*, *cgh-1* and other heterochronic genes that effect hypodermal cell-fate decisions.

			Percentage of Animals with Adult Alae Synthesis[a]							
			L3 Molt				L4 Molt			
	Strain	Genotype	No alae	Gapped	Comp.	(n)	No alae	Gapped	Comp.	(n)
1	N2	wild-type[b]	100	0	0	26	0	0	100	26
2	VT1273	*nhl-2(ok818)*[b]	100	0	0	28	0	3	97	70
3	VT2080	*nhl-3(tm2516); nhl-2(ok818) ncl-1(e1942) nhl-1 (gk15)*	-	-	-	-	0	3	97	72
4	CT8	*lin-41(ma104)*[c]	30	70	0	30	0	0	100	30
5	VT1914	*lin-41(ma104); nhl-2(ok818)*	100	0	0	30	0	0	100	20
6	VT1055	*alg-1(gk214)*[b]	-	-	-	-	0	34	66	33
7	VT2108	*alg-1(tm369)*[b]	-	-	-	-	0	61	39	28
8	VT1278	*nhl-2(ok818); alg-1(gk214)*[b]	-	-	-	-	26	64	10	27
9	VT2124	*nhl-2(ok818); alg-1(tm369)*[b]	-	-	-	-	71	19	10	31
10	VT2008	*ain-1(ku322)*[b]	-	-	-	-	0	52	48	33
11	VT2007	*nhl-2(ok818); ain-1(ku322)*[b]	-	-	-	-	85	15	0	34
12	VT1307	*mir-48(n4097)*[b]	-	-	-	-	0	0	100	26
13	VT1297	*nhl-2(ok818); mir-48(n4097)*[b]	-	-	-	-	11	85	4	26
14	VT1313	*mir-84(n4037)*[b]	-	-	-	-	0	0	100	27
15	VT1314	*nhl-2(ok818); mir-84(n4037)*[b]	-	-	-	-	0	0	100	22
16	VT1064	*mir-48(n4097); mir-84(n4037)*[b]	-	-	-	-	0	4	96	25
17	VT1298	*nhl-2(ok818); mir-48(n4097); mir-84(n4037)*[b]	-	-	-	-	57	40	3	30
18	VT1296	*mir-48 mir-241(nDf51)*[b]	-	-	-	-	0	58	42	33
19	VT1300	*nhl-2(ok818); mir-48 mir-241(nDf51)*[b]	-	-	-	-	88	12	0	33
20	VT1423	*mir-48 mir-241(nDf51); mir-84(n4037)*[b]	-	-	-	-	85	15	0	32
21	VT1425	*nhl-2(ok818); mir-48 mir-241 (nDf51); mir-84 (n4037)*[b]	-	-	-	-	80	20	0	35
22	VT1936	*cgh-1(ok492)*[b,d]	-	-	-	-	0	3	97	66
23	VT1999	*cgh-1(ok492); mir-48(n4097); mir-84(n4037)*[b,d]	-	-	-	-	96	4	0	27
24	VT2089	*nhl-2(ok818) cgh-1(ok492)*[d]	-	-	-	-	100	0	0	30

			Percentage of Animals with Adult Alae Synthesis[a]							
			L3 Molt				L4 Molt			
	Strain	Genotype	No alae	Gapped	Comp.	(n)	No alae	Gapped	Comp.	(n)
25	VT2115	*cgh-1(ok492); alg-1(tm369)*[d]	-	-	-	-	100	0	0	22

[a] Presence and quality of cuticular alae structures were assayed by Normarski DIC optics. Only one side of each animals was scored.

[b] Animals contain *maIs105* which expresses an adult specific, *col-19::GFP* reporter integrated on chromosome V near *mir-48* and *mir-241*.

[c] *lin-41(ma104)* animals display a relatively mild heterochronic phenotype and typically only produce a small patch of alae over one or two seam cells during the L3 molt. At the L4 molt, *lin-41 (ma104)* animals produce normal, continuous adult alae.

[d] Animals were derived from a parental strain containing *hT2-GFP* (I;III) chromosome that balances *cgh-1(ok492)* III and *nhl-2(ok818)* III.