

The TRIM-NHL protein NHL-2 is a Novel Co-Factor of the CSR-1 and HRDE-1 22G-RNA Pathways

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21 **ABSTRACT**

22 Proper regulation of germline gene expression is essential for fertility and maintaining species integrity.

23 In the *C. elegans* germline, a diverse repertoire of regulatory pathways promote the expression of

24 endogenous germline genes and limit the expression of deleterious transcripts to maintain genome

25 homeostasis. Here we show that the conserved TRIM-NHL protein, NHL-2, plays an essential role in

26 the *C. elegans* germline, modulating germline chromatin and meiotic chromosome organization. We

27 uncover a role for NHL-2 as a co-factor in both positively (CSR-1) and negatively (HRDE-1) acting

28 germline 22G-small RNA pathways and the somatic nuclear RNAi pathway. Furthermore, we

29 demonstrate that NHL-2 is a bona fide RNA binding protein and, along with RNA-seq data point to a

30 small RNA independent role for NHL-2 in regulating transcripts at the level of RNA stability.

31 Collectively, our data implicate NHL-2 as an essential hub of gene regulatory activity in both the

32 germline and soma.

INTRODUCTION

The conserved family of TRIM-NHL proteins have emerged as key regulatory points of gene expression to impact a variety of processes including stem cell self-renewal, developmental patterning and cellular differentiation in metazoans (Tocchini and Ciosk, 2015). TRIM-NHL proteins contain a N-terminal tripartite motif (TRIM), composed of a zinc finger RING domain, one or two B-box zinc finger motifs (distinct from the RING domain), and a coiled-coil region, in association with six C-terminal NHL motifs (Supplemental Fig. S1). This combination of domains enables TRIM-NHL proteins to modulate gene expression in versatile ways, ranging from the ubiquitination of protein targets (via the RING domain and associated B-box motifs) (Ikeda and Inoue, 2012) to regulating mRNA stability or translation via the NHL domain (Loedige, et al., 2013). However, the full repertoire of gene regulatory activities and the developmental specificity of such functions by TRIM-NHL proteins remains to be fully elucidated.

How TRIM-NHL proteins regulate specific mRNAs is only beginning to become clear. The prevailing notion has been that TRIM-NHL proteins interact with mRNA via interactions with other proteins. For instance, the *Drosophila* TRIM-NHL protein Brat was long thought to recognize maternal mRNAs via an interaction with the RNA binding protein Pumilio (Sonoda and Wharton, 2001). However, TRIM-NHL proteins have recently been shown to directly bind to RNA via NHL motifs (Laver, et al., 2015; Loedige, et al., 2015; Loedige, et al., 2014). For some TRIM-NHL proteins, such as Brat, this interaction occurs with a reasonable degree of sequence specificity, with a preference for U rich motifs, and leads to the post-transcriptional and/or translational regulation of target transcripts (Laver, et al., 2015; Loedige, et al., 2015; Loedige, et al., 2014). In addition to binding and regulating RNA directly, several TRIM-NHL proteins have been implicated in miRNA-mediated regulation of

transcripts, adding another layer of complexity to their gene regulatory capacity (Hammell, et al., 2009; Schwamborn, et al., 2009; Neumuller, et al., 2008).

NHL-2 is one of five members of the TRIM-NHL family in *C. elegans* (including NHL-1, 2, 3; LIN-41; NCL-1). Thus far, these paralogs have been shown to control pluripotency (LIN-41) (Tocchini, et al., 2014), oocyte growth and meiotic maturation (LIN-41) (Tsukamoto, et al., 2017; Spike, et al., 2014), and inhibit translation (NCL-1) (Yi, et al., 2015). In comparison, NHL-2 has been shown function the sex determination pathway (McJunkin and Ambros, 2017) and also acts as a co-factor in the *C. elegans* miRNA pathway where it is required for proper developmental timing and cell fate progression (Karp and Ambros, 2012; Hammell, et al., 2009). In the miRNA pathway, NHL-2 does not impact the biogenesis of miRNAs but exerts a positive influence on RNA Induced Silencing Complex activity (RISC, including small RNA or miRNA bound to the Argonaute effector, along with accessory proteins) (Hammell, et al., 2009). NHL-2 associates with the miRNA specific Argonaute effectors, ALG-1/2, and other miRISC co-factors, including AIN-1/GW182 and the DEAD-box RNA helicase CGH-1/DDX6 (Hammell, et al., 2009). Furthermore, loss of *nhl-2* does not broadly affect miRNA activity, but instead specifically influences miRISC activity associated with two miRNAs: *let-7* and *lsy-6* (Hammell, et al., 2009). Similarly, the NHL-2 ortholog TRIM32 in *M. musculus* and *H. sapiens* regulates a small number of miRNAs, including *let-7a*, while in *D. melanogaster*, Brat and Mei-P26 have also been shown to act broadly as miRNA co-factors (Loedige, et al., 2014; Schwamborn, et al., 2009; Neumuller, et al., 2008). Individual TRIM-NHL proteins exert positive or negative effects on the miRNA pathway, and thereby control developmental programs (Loedige, et al., 2014; Schwamborn, et al., 2009; Neumuller, et al., 2008). Although the functions of NHL-2 in the soma are linked to miRNA function, what role, if any, NHL-2 plays in the germline remains unclear.

77 In addition to miRNAs, the small RNA regulatory repertoire in *C. elegans* includes endogenous
78 small interfering RNAs (endo-siRNAs; 22G-RNAs, 26G-RNAs), and Piwi-interacting RNAs
79 (piRNAs/21U-RNAs), which are part of an elaborate surveillance system to regulate homeostasis of the
80 germline transcriptome (reviewed in (Billi, et al., 2014; Youngman and Claycomb, 2014)). The 22G-
81 RNA family of endo-siRNAs is the most functionally diverse class of germline small RNAs (named
82 22G because they are generally 22 nucleotides in length, and possess a 5'-triphosphorylated guanosine
83 residue) (Gu, et al., 2009). It has been proposed that 22G-RNAs are selectively loaded onto either the
84 Argonaute CSR-1 or the WAGO sub-family of AGOs, where they subsequently go on to fulfill distinct
85 gene-regulatory functions (de Albuquerque, et al., 2015; Phillips, et al., 2015; Phillips, et al., 2014;
86 Phillips, et al., 2012; Claycomb, et al., 2009; Gu, et al., 2009). The synthesis of 22G-RNAs relies on a
87 complex containing an RNA dependent RNA polymerase enzyme (RdRP; EGO-1 or RRF-1), in
88 association with the DEAD-box RNA helicase DRH-3 and the dual Tudor domain protein EKL-1 (Gu,
89 et al., 2009; Aoki, et al., 2007). Although it remains largely unclear how the 22G-RNAs are specified
90 for their different AGO effectors, one notable feature of the two pathways is that the CSR-1 22G-RNAs
91 are produced solely by the RdRP EGO-1, while the WAGO 22G-RNAs rely on both RRF-1 and EGO-
92 1 for their biogenesis (Phillips, et al., 2014; Phillips, et al., 2012; Claycomb, et al., 2009; Gu, et al.,
93 2009).

94 The CSR-1 22G-RNAs are required for normal chromosome organization in the germline and
95 embryos, likely due to genome-wide effects on chromatin and/or transcription (Wedeles, et al., 2013a).
96 CSR-1 associates with genomic loci of its gene targets, and recent data point to a role for the CSR-1
97 pathway in promoting the transcription of these germline genes trans-generationally (Cecere, et al.,
98 2014; Conine, et al., 2013; Seth, et al., 2013; Wedeles, et al., 2013b; Claycomb, et al., 2009), as well as

post-transcriptionally fine-tuning the expression of target genes in embryos via its slicer activity (Gerson-Gurwitz, et al., 2016). In contrast, the WAGO 22G-RNAs, namely those associated with WAGO-1 and HRDE-1, are required for silencing of endogenous pseudogenes, transposons, and protein-coding genes both at the transcriptional and post-transcriptional levels. These activities are largely downstream of the piRNA pathway, and function to recognize and silence foreign nucleic acid (Ashe, et al., 2012; Buckley, et al., 2012; Shirayama, et al., 2012; Gu, et al., 2009). Together, the CSR-1 and WAGO pathways provide an epigenetic memory of self (CSR-1) from non-self (WAGOs) nucleic acid and are critical for maintaining genomic and transcriptional integrity of the germline (Wedeles, et al., 2014; Conine, et al., 2013; Seth, et al., 2013; Wedeles, et al., 2013b; Claycomb, et al., 2009).

Here we characterize the role of NHL-2 in the germline and provide evidence that, in addition to its role in the somatic microRNA pathway, NHL-2 is a biogenesis factor in the CSR-1 and WAGO germline 22G-RNA pathways. Like other 22G-RNA biogenesis factors and AGOs, including CSR-1 and WAGO-1, NHL-2 localizes to P granules in the germline. Characterization of *nhl-2(ok818)* mutants reveals phenotypes consistent with loss of CSR-1 pathway activity, including: embryonic lethality, defects in oocyte chromosome organization, and aberrant accumulation of the repressive histone modification, H3K9me2 on germline autosome chromatin. We also found an unexpected role for NHL-2 in the nuclear RNAi pathway, and accompanying temperature-sensitive transgenerational fertility defect. Using an RNAi screen, we demonstrate that *nhl-2* genetically interacts with the 22G-RNA pathway components *drh-3*, *ekl-1*, *cde-1* and *csr-1*, and physically associates with CSR-1, HRDE-1 and DRH-3. High throughput sequencing of small RNAs in *nhl-2(ok818)* mutants reveals a depletion of 22G-RNAs for a subset of CSR-1 and WAGO target genes. Moreover, alterations in the

distribution of 22G-RNAs across CSR-1 target genes suggest a mechanism of NHL-2 action in 22G-RNA biogenesis, and implicate NHL-2 in distinct roles for germline versus somatic small RNA pathways. RNA binding assays demonstrate that NHL-2 is a bona fide RNA binding protein that specifically associates with U-rich sequences, and mRNA-seq experiments on *nhl-2(ok818)* mutants support the model that NHL-2 regulates a large number of somatic transcripts via this intrinsic RNA binding activity. Together, our results show that NHL-2 is a key factor in multiple facets of germline and somatic gene regulation.

RESULTS

NHL-2 is Required for Normal Germline Function and is Enriched in Germ Granules

While *nhl-2* mRNA is reported to be highly expressed in the *C. elegans* germline (Ortiz, et al., 2014; Reinke, et al., 2004) its function in this tissue remains relatively unknown. To determine if NHL-2 is required for fertility, we analyzed the brood size of wild-type and *nhl-2(ok818)* deletion null allele (Hammell, et al., 2009) animals at 20°C, 23°C and 25°C. At all three temperatures, compared to wild-type worms, *nhl-2(ok818)* displayed a significantly reduced brood size, and at 23°C and 25°C there was a significant increase in embryonic lethality (Fig. 1A, B). This temperature-dependent fertility defect has been described for several strong loss of function or null alleles of germline genes, including factors involved in small RNA pathways (Phillips, et al., 2012; Gu, et al., 2009; Van Wolfswinkel, et al., 2009; Wang and Reinke, 2008) and is likely to reflect temperature-sensitive processes in this tissue.

To determine if there were any gross morphological defects that would lead to infertility, we examined dissected one-day-old hermaphrodite germlines using DAPI staining with DIC and fluorescence microscopy. DIC microscopy revealed no overt morphological differences between *nhl-*

2(ok818) and wild-type germlines (data not shown). However, when we examined the organization of diakinetoc oocyte chromosomes in wild-type and *nhl-2(ok818)* animals via DAPI staining, we noted severe defects in *nhl-2(ok818)* mutants at all temperatures. While wild-type diakinetoc oocytes displayed six discrete DAPI bodies, indicative of homologous chromosome pairs, *nhl-2(ok818)* worms possessed a range of chromosomal abnormalities including aggregation into disorganized clumps, and greater than six DAPI bodies (Fig. 1C, D). Together these data indicate that NHL-2 is required for normal reproductive capacity and germline chromosome organization.

We next examined where and when NHL-2 is expressed in the germline. To do this, we raised antibodies against the N-terminus of NHL-2 and confirmed that the antibodies were specific for NHL-2 by immunostaining *nhl-2(ok818)* mutant germlines (Supplemental Fig. 2A). We then co-stained wild-type germlines for NHL-2 and CGH-1, a germline helicase with which NHL-2 was previously shown to interact (Hammell, et al., 2009). We found that NHL-2 co-localizes with CGH-1 in the gonad in both perinuclear germ granules, known as P granules in *C. elegans*, and in the cytoplasmic core of the syncytial germline (Fig. 1E). P granules are cytoplasmic aggregations of mRNA and protein that contribute to germ cell fate, and many RNA regulatory pathways, including those involved in small RNA pathways, localize to these structures (Voronina, et al., 2011). We also observed that NHL-2 localizes to P granules in early-stage embryos and was quickly lost from the somatic lineages after the 4-cell stage (Supplemental Fig. 2B and data not shown), consistent with a previous study (Hyenne, et al., 2008).

Given the interaction of NHL-2 and CGH-1 in the somatic miRNA pathway (Hammell, et al., 2009), we next examined whether either factor is required for the proper localization of the other factor. First, the absence of NHL-2 does not appear to affect P granule formation, as the P granule markers

PGL-1, CAR-1 and CGH-1 localized normally in *nhl-2(ok818)* mutants (Supplemental Fig. S2A and data not shown). CGH-1 is essential for fertility and localization of several P granule components (Arnold, et al., 2014; Sengupta and Boag, 2012; Boag, et al., 2008; Audhya, et al., 2005; Boag, et al., 2005), however in a *cgh-1* loss of function mutant NHL-2 localized normally, while CAR-1 localized to “sheet-like” structures in the gonad core (Supplemental Fig. S2C). These data suggest that although NHL-2 has similar germline localization pattern to CGH-1 and functions with CGH-1 in the soma, NHL-2 may contribute to germline gene regulation through different mechanisms.

***nhl-2* Genetically Interacts with the 22G-RNA Pathway**

To uncover any pathways in which NHL-2 may function to regulate germline development, we conducted a genome-wide RNAi screen for synthetic interactions (Davis, et al., 2017). Out of 11,511 genes screened, we identified 42 genes, that when knocked down in the *nhl-2(ok818)* null background resulted in strong synthetic phenotypes, including sterility, embryonic lethality and larval arrest. Among the candidates are genes encoding factors required for biogenesis of the 22G-RNAs. These include the poly(U) polymerase CDE-1, along with the RdRP complex proteins EKL-1 (a dual Tudor domain protein) and DRH-3 (a Dicer related helicase), which have been shown to physically interact (Gu, et al., 2009). CDE-1 was specifically implicated in the CSR-1 22G-RNA pathway, while DRH-3 and EKL-1 function in both CSR-1 and WAGO 22G-RNA pathways.

Notably, previous data demonstrated that CSR-1 pathway components display defects in diakinetik chromosome organization similar to *nhl-2(ok818)* mutants (Claycomb, et al., 2009; She, et al., 2009; Van Wolfswinkel, et al., 2009; Nakamura, et al., 2007). To more carefully assess the genetic interaction between *nhl-2(ok818)* and these 22G-RNA factors, we examined diakinetik oocyte defects

187 when we knocked down these genes in wild-type versus *nhl-2(ok818)* animals (Fig. 2A-E). Because of
 188 the link between *cde-1* and *csr-1*, we also examined *csr-1* in these assays. Diakinetic oocyte
 189 chromosomal organization was binned into four categories in our assays: 1) normal morphology, where
 190 6 pairs of homologous chromosomes were evident, 2) >6 chromosomes, where univalent (unpaired)
 191 chromosomes were present, 3) aggregated chromosomes, where clumping of chromosomes was
 192 observed, 4) enhanced aggregation, where chromosomes were tightly clumped. Consistent with
 193 previous results, we observed chromosome anomalies when *drh-3*, *csr-1*, *ekl-1* or *cde-1* were knocked
 194 down in wild-type animals (Fig. 2A-E). Importantly, knockdown of each 22G-RNA factor in *nhl-*
 195 *2(ok818)* mutants resulted in significantly enhanced chromosomal aggregation (Fig. 2A-E) at both
 196 20°C and 25°C. Furthermore, and consistent with these results, we observed an increase in the
 197 embryonic lethality of *nhl-2(ok818)* animals depleted of CSR-1 pathway factors, pointing to a strong
 198 synthetic interaction (Supplemental Fig. S3).

199 Another phenotype common among CSR-1 22G-RNA pathway co-factors is the abnormal
 200 accumulation of the repressive histone modification, Histone H3, Lysine 9 di-methylation (H3K9me2)
 201 on autosomes (She, et al., 2009). Normally, chromosomes that do not possess a pairing partner during
 202 meiosis (including the male X chromosome) are enriched for H3K9me2 and transcriptionally silenced
 203 in a process termed Meiotic Silencing of Unpaired Chromatin (MSUC). Loss of the CSR-1 pathway
 204 leads to aberrant accumulation of H3K9me2 on autosomes and results in homologous pairing defects.
 205 Given the synthetic interaction of *nhl-2* with factors in the CSR-1 22G pathway, we examined if *nhl-*
 206 *2(ok818)* mutants displayed altered H3K9me2 distribution. Using an antibody specific for H3K9me2,
 207 we immunostained germlines from wild-type males depleted for *drh-3* or *csr-1* by RNAi and compared
 208 them to *nhl-2(ok818)* males (Fig. 2F). Consistent with previous reports, depletion of *drh-3* or *csr-1* in

209 wild-type males resulted in abnormal accumulation of H3K9me2 (Wang and Reinke, 2008).
 210 Interestingly, *nhl-2(ok818)* males also display abnormal H3K9me2 accumulation in a similar manner to
 211 that observed in *drh-3(RNAi)* and *csr-1(RNAi)* animals. We quantified the defects in H3K9me2 patterns
 212 as follows: 1) normal, where each germ cell displayed one strong H3K9me2 signal (as seen in wild-
 213 type), 2) elevated, where germ cells displayed one or more H3K9me2 markings that were greater than
 214 that of wild-type germ cells, 3) dispersed, where H3K9me2 markings were distributed on various
 215 chromosomes within a germ cell (Fig. 2G). Abnormal autosomal H3K9me2 enrichment in *nhl-*
 216 *2(ok818)* germ cells suggests that *nhl-2(ok818)* mutants have errors in MSUC and meiotic synapsis
 217 similar to *drh-3* or *csr-1*, and are consistent with a role for NHL-2 in the CSR-1 pathway.

218

219 **NHL-2 is required for nuclear RNAi inheritance**

220 Multiple components of small RNA pathways have been shown to be required for nuclear
 221 RNAi and germline immortality (Spracklin, et al., 2017; Weiser, et al., 2017; Ashe, et al., 2012;
 222 Buckley, et al., 2012), therefore, we next examined if NHL-2 is required for these pathways. To
 223 examine nuclear RNAi, we focused on the polycistronic pre-mRNA that encodes the non-essential gene
 224 *lir-1* and the essential gene *let-26* (Bosher, et al., 1999). In wild-type animals, RNAi targeting *lir-1*
 225 results in the silencing of the polycistronic pre-mRNA by the nuclear RNAi pathway, resulting in *let-26*
 226 phenotypes of larval arrest and lethality (Fig. 3A) (Bosher, et al., 1999). Interestingly, *nhl-2(ok818)*
 227 animals were resistant to *lir-1* RNAi and closely resembled *nrde-2* and *rde-4* nuclear RNAi mutants
 228 (Fig. 3B). Some RNAi factors, such as *hrde-1*, have a temperature-sensitive transgenerational mortal
 229 germline (Mrt) phenotypes (Spracklin, et al., 2017; Weiser, et al., 2017; Ashe, et al., 2012; Buckley, et
 230 al., 2012), therefore, we tested if *nhl-2(ok818)* also displays this phenotype. Consistent with the Mrt

phenotype, after 10-12 generations *nhl-2(ok818)* worms grown at 25°C became sterile (Fig. 3C) while wild-type worms remained fertile. We next examined if shifting *nhl-2(ok818)* worms grown at 25°C to 20°C could rescue the decline in fertility in subsequent generations. As has been demonstrated for other Mrt mutants (Spracklin, et al., 2017; Ni, et al., 2016), when the F1 generation of *nhl-2(ok818)* worms were moved at the L1 stage to 20°C it took 3 generations for the brood size to recover to the normal *nhl-2(ok818)* level (Fig. 3D). Similarly, embryonic lethality took a generation to recover to the *nhl-2(ok818)* 20°C level (Fig. 3E). These findings are consistent the Mrt phenotype observed in mutants in the nuclear RNAi pathway and suggests the decreased brood size observed at 25°C is not simply the result of DNA damage. Together these data are consistent with NHL-2 participating in nuclear RNAi and multigenerational epigenetic inheritance pathways.

NHL-2 Physically Interacts with CSR-1 and HRDE-1 Pathway Proteins

Because NHL-2 had previously been shown to physically interact with miRNA Argonautes, we decided to test for any association with the 22G-RNA-associated AGOs CSR-1 and HRDE-1. We first performed co-Immunoprecipitation of endogenous NHL-2 and probed for CSR-1 by western blotting. Indeed, we found that CSR-1 associates with NHL-2 by co-IP in adult hermaphrodites (Fig. 4A). We then moved on to test for interactions with other members of the CSR-1 pathway by similar experiments, and found an interaction between NHL-2 and the RdRP complex helicase, DRH-3 (Gu, et al., 2009) (Fig. 4B). To test for additional protein interactions by a separate method, we incubated purified GST-tagged full-length NHL-2 with protein lysate and determined whether NHL-2 was able to interact with components of the 22G-RNA biogenesis machinery by western blotting. In these experiments, we observed that GST-NHL-2 associated with both CSR-1 and HRDE-1 from whole adult

253 worm lysates, but not WAGO-1, or other components of the EGO-1, RRF-1 or EKL-1 (Fig. 4C, data
254 not shown). These data indicate that NHL-2 associates with CSR-1 and HRDE-1 pathway proteins at
255 multiple points of pathway activity (with DRH-3 of the RdRP complex and with AGO effectors of the
256 RISC).

257 The C-terminal RING domain of TRIM-NHL proteins is often associated with E3 ubiquitin
258 ligase activity and proteasome-mediated protein turnover. To determine whether NHL-2 functions as
259 an E3 ubiquitin ligase that targets proteins of the CSR-1 pathway for degradation, we examined CSR-1
260 expression in wild-type and *nhl-2(ok818)* one-day-old adult animals. We observed comparable levels
261 of both CSR-1 and DRH-3 protein in wild-type and *nhl-2(ok818)* animals (Supplemental Fig. S4A and
262 data not shown for DRH-3), suggesting that the association of NHL-2 and CSR-1 is not related to
263 potential E3 ubiquitin ligase activity by NHL-2.

264

265 **NHL-2 Is Required for Maintaining the Steady-State Levels of 22G-RNAs**

266 To explore the role of *nhl-2* in the biogenesis or stability of the 22G-RNAs, we conducted small
267 RNA high throughput-sequencing of wild-type and *nhl-2(ok818)* animals at 20°C and 25°C
268 (Supplemental Table S1, S3). The overall size and first nucleotide distributions of small RNA species
269 in *nhl-2(ok818)* mutants are consistent with the wild-type controls at both temperatures (Supplemental
270 Fig. S5, S6). However, when we began to examine particular classes of small RNAs in each genotype
271 and at the different temperatures, we observed some interesting differences.

272 First, we observed overall changes in some small RNA populations in both wild-type and *nhl-*
273 *2(ok818)* mutants at the higher temperature. There was an overall decrease in 21U-RNAs at 25°C
274 compared with 20°C, for both wild-type and *nhl-2(ok818)* mutants (Supplemental Figs. S5, S4, S6).

275 This may reflect the temperature-dependent nature of the production or stability of these small RNAs.
 276 Notably, many mutants in the piRNA and 26G-RNA pathways exhibit temperature sensitive fertility
 277 defects that appear to be consistent with an overall decline in these small RNA populations at higher
 278 temperatures. 21-23nt siRNA levels (which encompass the 22G-RNA category) increased slightly in
 279 both wild-type and *nhl-2(ok818)* mutants at 25°C compared to 20°C, while miRNA levels were
 280 relatively unchanged in both strains between temperatures (Supplemental Figs. S5, S6, S7).

281 Next, we compared wild-type and *nhl-2(ok818)* mutant small RNA populations at each
 282 temperature. We observed a consistent decrease in 22G-RNA and 26G-RNA populations in *nhl-*
 283 *2(ok818)* mutants relative to wild-type at 25°C (Supplemental Figs. S5, S6, S7). Concomitant with the
 284 22G-RNA decreases, we observed an increase in the proportion of miRNAs in *nhl-2(ok818)* mutants.
 285 While it is possible that this change in miRNAs is biologically meaningful, it is more likely due to a
 286 “filling in” of the cloning space when small RNA populations are expressed as a proportion of the total
 287 reads, as previous reports observed little change in miRNA levels in *nhl-2(ok818)* mutants (Hammell,
 288 et al., 2009). Finally, we observed decreases in the 21U-RNA populations of *nhl-2(ok818)* mutants
 289 relative to wild-type at 20°C, which are not evident at 25°C.

290 Because of their abundance and the link between NHL-2, CSR-1, and the RdRP complex that
 291 synthesizes 22G-RNAs (via an interaction between NHL-2 and DRH-3), we examined this class of
 292 small RNAs in greater depth (Fig. 5A, B, Supplemental Table S2). We observed that 573 and 724
 293 genes display a two-fold or greater depletion of 22G-RNAs in *nhl-2(ok818)* mutants relative to wild-
 294 type at 20°C and 25°C respectively, with 381 genes in common between the two temperatures (the top-
 295 right Venn-pie diagram in Figure 5A).

296 The genes depleted of 22G-RNAs in *nhl-2(ok818)* mutants at 20°C are enriched in WAGO-1
 297 and HRDE-1 target genes (Fig. 5A), comprising 36.5% (209/573; hypergeometric test q-value or $q =$
 298 $9.6E-80$) and 36.6% (210/573; $q = 2.1E-83$), respectively, with 66 genes shared between the two
 299 WAGOs (WAGO-1 targets are as defined in (Gu, et al., 2009), and HRDE-1 targets are as defined in
 300 (Shirayama, et al., 2012). Consistent with this result, 74.5% (427/573; $q = 1.6E-296$) of these genes
 301 were depleted of 22G-RNAs in a mutant strain that carries mutations in twelve *wago* (12-fold *wago*
 302 mutant including mutations in *hrde-1* and *wago-1* (Gu, et al., 2009)). Notably, 33% (189/573; $q = 3.7E-$
 303 5) of these genes are CSR-1 targets, with only 17 of these genes overlapping with HRDE-1 or WAGO-
 304 1 targets (CSR-1 targets are as defined in (Tu, et al., 2015)).

305 Similar to the results at 20°C, 29.3% of the 724 genes depleted of 22G-RNAs in *nhl-2(ok818)*
 306 mutants at 25°C are CSR-1 target genes (212/724; $q = 3.7E-2$), while 33.7% and 37.2% are WAGO-1
 307 and HRDE-1 targets (244/724 and 269/724; $q = 2.1E-85$ and $4.7E-110$; with 139 genes in common).
 308 Nearly 78% (563/724, $q < 1E-300$) of the genes depleted of 22G-RNAs in *nhl-2(ok818)* mutants at
 309 25°C overlap with genes depleted of small RNAs in the 12-fold *wago* mutant. Finally, the majority of
 310 genes depleted of 22G-RNAs in *nhl-2(ok818)* mutants at either temperature significantly overlap with
 311 genes depleted of 22G-RNAs in RdRP complex mutants, *ekl-1(tm1599)*, *ego-1(om97)*, and *drh-*
 312 *3(ne4253)* (all q -values $< 7.3E-195$) (Fig. 5A). The overlap between genes depleted of 22G-RNAs in
 313 *nhl-2(ok818)* mutants and the three well characterized germline small RNA pathways, coupled with the
 314 overlapping phenotypes of *nhl-2(ok818)* and *ago* mutants point to a role for NHL-2 in germline small
 315 RNA pathways overall.

316 The *glp-4(bn2)* small RNA data set reveals the complete repertoire of germline genes targeted
 317 by 22G-RNAs (Tu, et al., 2015; Gu, et al., 2009), as these mutants do not possess significant germline

318 tissue, and 5,971 genes show a two-fold or greater depletion of small RNAs. Consistent with the role of
 319 NHL-2 in fertility and germ cell development (Fig. 1, 2), 83.8% (480/573, $q = 2.6E-166$) and 83.4%
 320 (604/724 $q = 3.9E-209$) of the genes depleted of 22G-RNAs in *nhl-2(ok818)* mutants at 20°C and 25°C,
 321 respectively, are also depleted of 22G-RNAs in *glp-4(bn2)* mutants (Fig. 5A). These data indicate that
 322 the genes depleted of 22G-RNAs in *nhl-2(ok818)* mutants are targeted by 22G-RNAs in the germline.
 323 Further comparison with the male- and female-specific gonad gene expression data indicated that
 324 58.4% (423/724; q -value = 0.0128) and 61.6% (353/573; q -value = $1E-4$) of the genes depleted of 22G-
 325 RNAs in *nhl-2(ok818)* mutants at 25°C and 20°C respectively overlap with genes generally expressed
 326 in the gonad (meaning that they are expressed in both the spermatogenic or oogenic gonads) (Fig. 5B).
 327 We observed a subtle, but statistically significant enrichment for oogenic genes in the *nhl-2(ok818)*
 328 depleted gene sets (16.4%, 119/724 genes at 25°C, $q = 2.2E-11$, 22.5%, 129/573 genes at 20°C, q -value
 329 = $2.7E-24$). This observation may simply be reflective of the developmental stage from which the
 330 samples were prepared (young adults undergoing oogenesis), but is also consistent with oogenesis
 331 defects we observed in *nhl-2(ok818)* mutants.

332

333 **NHL-2 Is Required for 22G-RNA Coverage at the 5' Portion of CSR-1 Target Genes**

334 The decrease in steady-state levels for a subset of 22G-RNAs in *nhl-2(ok818)* mutants could
 335 indicate a defect in 22G-RNA synthesis or stability. Given the link between NHL-2 and the RdRP
 336 complex, we hypothesized that NHL-2 could affect 22G-RNA synthesis to a greater extent than
 337 turnover. Thus, we performed a metagene analysis to examine the distribution of 22G-RNAs across the
 338 gene body (Fig. 5C-E, Supplemental Fig. S8). In wild-type worms, 22G-RNAs are distributed across
 339 the entire length of WAGO target genes and are present in greater abundance than CSR-1 22G-RNAs.

340 Conversely, CSR-1 target genes generally have fewer 22G-RNAs targeting them overall, with a slight
341 bias in small RNA coverage toward the 5' end. In *nhl-2(ok818)* mutants at 25°C relative to wild-type,
342 we noted that the distribution of 22G-RNAs at WAGO targets was generally unchanged over the entire
343 locus.

344 For CSR-1 target genes, the distribution of 22G-RNAs in *nhl-2(ok818)* mutants was distinct
345 from that in wild-type worms. At both 20°C and 25°C, there was a significant decrease in the
346 abundance of 22G-RNAs over the 5' half of the gene in *nhl-2(ok818)* mutants relative to wild-type
347 controls (at 20°C, there was a 33.6% reduction in 22G-RNAs, t-test p-value = 1.4E-79; at 25°C, there
348 was a 15.5% reduction in 22G-RNAs, t-test p-value = 1.77E-9). Examined a different way, the centroid
349 of the 22G-RNA distribution for the group of CSR-1 target genes shifts by 5.77% of the metagene
350 length towards the 3' end in *nhl-2(ok818)* mutants relative to the wild-type at 25°C (Wilcoxon rank-
351 sum test p-value = 3.67E-29; Fig. 5C, Supplemental Fig. S8). These data suggest that NHL-2 may
352 influence the activity and/or processivity of the RdRP complex.

353 Because our small RNA results indicated a possible role for NHL-2 in RdRP complex
354 processivity or activity, and we had observed genetic and physical interactions between NHL-2 and the
355 RdRP complex via DRH-3, we asked whether NHL-2 was required for the formation or stability of the
356 RdRP complex. Therefore, we tested whether the CSR-1 RdRP complex, as measured by an
357 association between the key components DRH-3 and EGO-1, could properly form in the absence of
358 NHL-2. To answer this question, we immunoprecipitated DRH-3 and probed for EGO-1 and EKL-1,
359 three key components of the CSR-1 RdRP complex, and found that the association between DRH-3,
360 EGO-1, and EKL-1 was maintained in the absence of NHL-2 (Supplemental Fig. S4). Thus, NHL-2 is
361 not required for the formation or maintenance of the RdRP complex, and may play a different role in

the biogenesis of 22G-RNAs, possibly in the handoff of newly synthesized 22G-RNAs to CSR-1 (because of its physical association with both CSR-1 and DRH-3), aiding the assembled RdRP complex in moving along the RNA template, or in the selection of particular mRNAs as templates for 22G-RNA synthesis.

The NHL Domain of NHL-2 Binds RNA

We went on to explore a role for NHL-2 in directly interacting with mRNA transcripts. The NHL domain of the TRIM-NHL protein Brat from *D. melanogaster* was recently shown to be a sequence-specific RNA binding protein, which suggests other NHL domain proteins may also bind directly to RNA (Loedige, et al., 2014). To determine whether the C-terminal NHL domain of NHL-2 binds RNA, we first generated a structural homology model based on the crystal structure of Brat (Edwards, et al., 2003). This modeling of the NHL domain revealed the canonical six-bladed "propeller" characteristic of this domain. It also showed that much of the surface of the protein was positively charged and therefore would likely interact with negatively charged RNA molecules (Fig. 6A). To explore if the NHL domain of NHL-2 binds to RNA we performed the RNAcompete *in vitro* binding assay (Ray, et al., 2013; Ray, et al., 2009). We expressed and purified a GST-tagged NHL domain of NHL-2 and incubated it with a complex pool of 240,000 30-41mer RNAs. RNAs that co-purified with the GST-tagged NHL domain were identified by microarrays and revealed a strong binding preference for U-enriched RNAs, with a core consensus of UUUU, and preference for U residues 5' and 3' to the core (Fig. 6B). We next examined the binding affinity of the NHL domain for a 5'-Fluorescein labelled 17mer poly-U RNA oligonucleotide using fluorescence anisotropy. In these experiments, GST-tagged NHL domain at a range of concentrations was mixed with poly-U RNA

oligonucleotide and allowed to reach equilibrium. These experiments yielded an equilibrium dissociation constant of $K_D = 0.41 \pm 0.02 \mu\text{M}$, and were consistent with a one-site binding model (Fig. 6C). These data strongly suggest that NHL-2 is a bona fide RNA-binding protein with an ability to bind U rich sequences.

Steady State mRNA Levels are Altered Independent of Small RNA Levels in *nhl-2(ok818)* Mutants

Because of its link to multiple small RNA pathways and its capacity to bind RNA, we asked whether NHL-2 plays a role in transcript regulation. To test this, we performed mRNA-seq in wild-type (N2) and *nhl-2(ok818)* adult animals at both 20°C and 25°C, with three biological replicates each (Fig. 7A, Supplemental Table S3). At 20°C, we observed less extensive changes in steady-state mRNA levels in *nhl-2(ok818)* mutants, with 1,014 genes increased and 1,630 genes decreased by two-fold or greater. This is in contrast to 25°C, where we identified 3,554 genes with two-fold or greater increases in steady-state mRNA levels, and 4,370 genes with two-fold or greater decreased steady-state mRNA levels in *nhl-2(ok818)* mutants relative to wild-type (Fig. 7A). There was a significant overlap of genes up-regulated in *nhl-2(ok818)* between the two temperatures (643 genes; q-value = 6.2E-249). Similarly, there was a significant overlap between down-regulated genes at both temperatures (1,326 genes; q-value < 1E-300). We then went on to examine the genes with altered expression in more detail.

Based on the previously described role for NHL-2 with *let-7* and *lisy-6* in the miRNA pathway, we first asked whether predicted targets of these particular miRNAs were de-repressed upon loss of *nhl-2* (Fig. 7B). Although several lines of data previously suggested that NHL-2 functioned with miRISC in the translational regulation of targets, no genome-wide transcriptome data in *nhl-2(ok818)*

mutants were available to test the possibility that NHL-2 and miRISC could impact targets via mRNA stability or turnover. Using TargetScan (Worm Release 6.2, June 2012), we identified 162 predicted targets of *lsy-6*, and 126 predicted targets of the *let-7* family of miRNAs. Only 33 of the 162 predicted *lsy-6* target mRNAs and 29 of the 126 *let-7* family predicted targets were up-regulated in *nhl-2(ok818)* mutants at 25°C. Examining the data the other way around, we asked if the genes up-regulated in *nhl-2(ok818)* mutants were enriched for *let-7* or *lsy-6* predicted targets and found no correlation (data not shown). Overall, these data indicate that miRNA target genes are not regulated by NHL-2 at the level of transcript abundance or stability, and instead, NHL-2 is likely to exert a predominantly translational mode of regulation on these genes.

We next asked whether the genes with altered 22G-RNA levels in *nhl-2(ok818)* mutants were differentially expressed (Fig. 7C, Supplemental Fig. S7). First, we overlapped the sets of genes depleted of 22G-RNAs in *nhl-2(ok818)* mutants at either temperature and were surprised to find only modest effects overall. Of the 573 genes depleted of 22G-RNAs at 20°C, 78 displayed increased steady-state mRNA levels (13.6%; 78/573 genes; q-value = 3.3E-14) and 11 had decreased steady-state mRNA levels (1.9%; 11/573: not significant). At 25°C, 724 genes showed depleted 22G-RNA levels, and of these 210 were up-regulated (29%; 210/724, q-value = 1.8E-13), while 81 were down-regulated (11.2%; 81/724: not significant). Thus, overall, genes displaying altered 22G-RNA levels were not extensively affected at the mRNA level by the loss of *nhl-2*, and the fraction that were affected at the mRNA level tended to be repressed by NHL-2 under wild-type conditions. Overall, these data are consistent with a role for NHL-2 in the biogenesis, but not necessarily the effector steps, of a subset of 22G-RNAs. These data could also point to a role for NHL-2 in regulating the translation of this subset

427 of germline small RNA target genes for which the 22G-RNA levels are altered in *nhl-2(ok818)*
428 mutants, perhaps in a manner similar to the miRNA pathway.

429 Next, we examined the steady state levels by evaluating the levels of CSR-1 or WAGO pathway
430 target genes in *nhl-2(ok818)* mutants, independent of any changes in 22G-RNA levels. In accordance
431 with the opposing roles of the WAGO and CSR-1 pathways in germline gene regulation, we anticipated
432 that the WAGO targets with decreased 22G-RNAs would display increased mRNA levels, while the
433 CSR-1 targets with decreased 22G-RNAs would display decreased mRNA levels in *nhl-2(ok818)*
434 mutants. We observed that a statistically significant subset of WAGO-1 target genes (18%; 141/1718
435 genes; q-value = 9.9E-8) were de-repressed at 20°C, and 291 out of 1718 WAGO-1 target genes were
436 de-repressed at 25°C (16.9%; q = 0.6790). HRDE-1 target genes also tended to be up-regulated in *nhl-*
437 *2(ok818)* mutants at both temperatures (141/1661; 8.5% q-value = 9.9E-9 at 20°C, 335/1661; 20.2%, q-
438 value = 9.9E-2 at 25°C). These results are consistent with a cooperative role for NHL-2 in the
439 repression of WAGO target genes, albeit in a small RNA-independent manner.

440 Unexpectedly, CSR-1 target mRNAs were significantly up-regulated *nhl-2(ok818)* mutants, and
441 this effect occurred specifically at 25°C (2,025/4932; 41%, q-value < 1E-300). Similarly, when
442 examined the behavior of total set of genes targeted by germline small RNAs using the *glp-4(bn2)*
443 mutant, we found a strong overlap between genes depleted of small RNAs in the *glp-4(bn2)* mutant and
444 genes up-regulated in *nhl-2(ok818)* mutants specifically at 25°C (60%; 2155/3554, q-value < 1E-300)
445 (Fig. 7C). These results are consistent with a small RNA-independent role for NHL-2 in the repression
446 of CSR-1 22G-RNA targets, and this activity is antagonistic to the action of CSR-1. Moreover, the
447 functions of NHL-2 in regulating germline small RNA pathway target genes, especially those of the
448 CSR-1 pathway, appear to be particularly important at high temperature (25°C) or perhaps other

stressful conditions. Overall, the changes in steady-state mRNA levels for germline 22G-RNA pathway target genes point to a role for NHL-2 in regulating transcripts via its intrinsic RNA binding capacity.

Loss of *nhl-2(ok818)* Impacts Genes Involved in Gametogenesis, Signaling, and Chromosome Organization

In an attempt to link alterations in gene expression to changes in phenotype for *nhl-2(ok818)* mutants, we next compared the *nhl-2(ok818)* transcriptome data to spermatogenic versus oogenic gonad transcriptomes (Ortiz, et al., 2014) (Fig. 7D). First, we found that a large fraction of genes that were mis-expressed in *nhl-2(ok818)* mutants at both temperatures were represented in total gonad transcriptomes (20°C up-regulated genes 41.1%, 417/1014, q-value = 1; 20°C down-regulated genes 69.4%, 1131/1630, q-value = 7.2E-46; 25°C up-regulated genes 75.9%, 2696/3554, q-value = 5.0E-216; 25°C down-regulated genes 56.8%, 2482/4370, q-value = 3.1E-9), indicating that most of the mis-regulated transcripts are expressed in the germline. Next, we found that genes up-regulated in *nhl-2(ok818)* mutants at 25°C were significantly enriched for gender neutral and oogenesis-associated transcripts (46.1%, 1637/3554; 25.2%, 894/3554; hypergeometric test q-values = 3.8E-100, and 2.8E-259, respectively), while, for those genes up-regulated at 20°C, only oogenesis-associated transcripts were enriched (14%, 142/1014; hypergeometric test q-value = 9.2E-8). Interestingly, down-regulated genes in *nhl-2(ok818)* mutants at both 20°C and 25°C were enriched for spermatogenesis-associated transcripts (47%, 766/1630; 35.6%, 1556/4370; hypergeometric test q-value = 1.8E-265 and <1E-300, respectively). These data point to an overall misregulation of the germline transcriptome that leads to defects gametogenesis and decreased fertility.

470 Given that NHL-2 and CGH-1 have been shown to physically interact, we next compared the
471 CGH-1 RNA-IP/microarray data with the *nhl-2(ok818)* transcriptome data (Fig. 7D). In the germline,
472 CGH-1 is required to protect specific maternal mRNAs (which overlap with those enriched in the
473 oogenesis and gender neutral transcriptomes) from degradation, and is also involved in translational
474 regulation of some transcripts(Boag, et al., 2008). We observed a small, but significant, enrichment
475 between CGH-1-associated and up-regulated transcripts in *nhl-2(ok818)* mutants at 25°C, (12.9%,
476 458/3554; hypergeometric test q-values = 4.2E-76). These data suggest an antagonistic role between
477 NHL-2 and CGH-1 in the regulation of the stability of these transcripts. To determine if there was any
478 interaction with NHL-2 and other described germline RNP complexes we examined mRNA targets of
479 the cytoplasmic poly(A) polymerase GLD-2 targets and the RNA-binding proteins OMA-1 and LIN-
480 41, all three critical for post-transcriptional regulation of the oocyte-to-embryo transitions (Tsukamoto,
481 et al., 2017). Similar to CGH-1-associated transcripts, GLD-2 target mRNAs (16.5%, 588/3554, q-
482 value = 1.0E-207) and OMA-1 (20.4%, 725/3554, q-value = 7E-67) and LIN-41-enriched transcripts
483 (13%, 462/3554, q-value = 8E-80) had a small but significant enrichment compared to up-regulated
484 transcripts in *nhl-2(ok818)* mutants at 25°C. This is consistent with the strong overlap between genes
485 expressed during oogenesis and those upregulated in *nhl-2(ok818)* mutants at 25°C. These data suggest
486 that NHL-2 does not regulate the majority of mRNAs found in the key pathways governing the oocyte-
487 to-embryo transitions, however, we cannot rule out any translational effects of NHL-2 in this context.

488 When we performed Gene Ontology (GO) analysis, we found that the genes that are down-
489 regulated in *nhl-2(ok818)* mutants at 20°C and 25°C shared consistent sets of GO terms, and are
490 strongly enriched in cuticle/collagen proteins, kinases and phosphatases, and spermatogenesis proteins
491 (FDR = 6.1E-20 *protein kinase, core*, 7.9E-25 *phosphatase activity*, and 1.5E-31 *major sperm protein*,

492 respectively). Genes that are up-regulated in *nhl-2(ok818)* mutants at 20°C are weakly enriched in
 493 signaling molecules and oxidative metabolism (FDR = 1.4E-12 *signal peptide*, and 2.8E-3 *oxidation*
 494 *reduction*, respectively), and differ from the GO terms observed for the genes up-regulated at 25°C,
 495 which were enriched for cell cycle, *kinetochore*, *RNA-binding* and *DNA replication* and *damage repair*
 496 (FDR = 5.5E-35 cell cycle, 3.7E-12 *kinetochore*, 1.1E-16 *RNA binding*, and 3.7E-14 *DNA replication*
 497 and 9.6E-10 damage repair GO terms. In addition to analyzing the complete sets of up-regulated or
 498 down-regulated genes in the *nhl-2(ok818)* mutants, we also performed GO analysis on sets of
 499 transcripts that were expressed in the gonad, and observed comparable results (data not shown).
 500 Collectively, our data point to a role for NHL-2 in regulating the stability of a large fraction of
 501 gonad/germline transcripts that are involved in spermatogenesis, cellular signaling cascades, cuticle
 502 formation, and kinase/phosphatase activities, and chromosome organization. NHL-2 impacts these
 503 mRNAs both positively and negatively, and likely utilizes the intrinsic RNA binding properties of its
 504 NHL domain, perhaps in association with other protein binding partners, to do so.

505

506 **DISCUSSION**

507 TRIM-NHL proteins have been shown to play a variety of crucial roles in the context of the
 508 proliferation versus differentiation decision in metazoans. With modular and varied domains, TRIM-
 509 NHL proteins can function as E3 ligases as well as sequence-specific RNA binding proteins
 510 (Schwamborn, et al., 2009; Kudryashova, et al., 2005). TRIM-NHL proteins also engage the miRNA
 511 pathway, whereby, remarkably, their functions relate to only a few miRNAs, and impact the efficacy of
 512 the RISC both positively and negatively. With their intrinsic RNA binding activity, TRIM-NHL

513 proteins could regulate RNA directly or function at various steps and in virtually any small RNA
514 pathway.

515 NHL-2 is one of five TRIM-NHL proteins in *C. elegans* (paralogs include NHL-1, -3, LIN-41,
516 and NCL-1). NHL-2 has been shown to modulate miRISC via two specific miRNAs, *let-7* and *lys-6*,
517 which act in the soma to regulate developmental timing and cell fate transitions in multiple tissues.
518 Recently it was also shown that NHL-2 is also required for sex determination, although the mechanism
519 is unclear (McJunkin and Ambros, 2017). In spite of these intriguing roles for NHL-2 in the embryo
520 and soma, little is known about its functions in germline development. Here, we set out to explore a
521 role for NHL-2 in the germline and in germ cell development. We found that NHL-2 is required for
522 proper germline chromatin organization and wild-type levels of fertility at high temperatures and for
523 the somatic nuclear RNAi pathway. We also identified the AGOs CSR-1 and HRDE-1 and the RdRP
524 component DRH-3 as genetic and physical interactors of NHL-2. High throughput sequencing of small
525 RNA populations in *nhl-2(ok818)* mutants revealed an additional role for NHL-2 in the WAGO-1, and
526 HRDE-1 22G-RNA pathways, but as previous data suggested, little biologically meaningful
527 perturbation in the overall miRNA population. Binding assays confirm that NHL-2 is a bona fide RNA
528 binding protein, and examination of the mRNA transcriptome by mRNA-seq points to NHL-2 as a
529 post-transcriptional regulator of a substantial set of mRNAs involved in signaling, phosphorylation and
530 transcription independent of its small RNA activities. Together, our data, implicate NHL-2 as a
531 regulator of mRNA stability for a significant portion of the genome, a likely translational regulator of
532 miRNA targets, and a biogenesis factor and/or possible translational regulator of targets in the CSR-1
533 and WAGO 22G-RNA pathways.

534 We first identified a link between NHL-2 and the CSR-1 pathway by genetic and phenotypic
 535 studies, in which loss of CSR-1 pathway factors enhanced the aggregation of diakinetoc oocyte
 536 chromosomes in *nhl-2(ok818)* mutants. Loss of *nhl-2* also led to increased levels of H3K9me2 in
 537 pachytene germline nuclei and a spreading of this heterochromatin modification onto autosomes, where
 538 it is not normally observed. This phenotype is consistent with loss of CSR-1 pathway members,
 539 providing another phenotypic link between NHL-2 and the CSR-1 pathway. At this time, we do not
 540 entirely understand why this phenotype emerges in *nhl-2* or *csr-1* pathway mutants. It is possible that
 541 CSR-1 is not properly recruited to its target genes, due to mis-regulation of CSR-1 target transcripts in
 542 *nhl-2(ok818)* mutants. This, in turn, could disrupt the formation or maintenance of euchromatin at these
 543 loci and allow for the mis-direction of chromatin modifiers throughout the genome, as observed in *csr-*
 544 *1* mutants (Christopher Wedeles and Julie Claycomb, unpublished results). This leads to the aberrant
 545 accumulation of histone modifications throughout the genome, which could impact chromosome
 546 structure. Future ChIP-seq studies for histone modifications and CSR-1 recruitment in *nhl-2(ok818)*
 547 mutants will enable us to address this possibility. Alternatively, and based on the GO analysis, this
 548 chromosome organization defect could result indirectly from alterations in the levels of key transcripts
 549 associated with chromosome organization and metabolism, as has been proposed for CSR-1 (Gerson-
 550 Gurwitz, et al., 2016).

551 It was somewhat surprising to observe little correlation between the genes depleted of 22G-
 552 RNAs and genes with altered mRNA levels in *nhl-2(ok818)* mutants. Based on the known regulatory
 553 functions of these pathways we expected there would be a slight decrease in the level of CSR-1 target
 554 genes for which the 22G-RNAs were depleted, and an increase in the set of genes targeted by WAGO-1
 555 or HRDE-1 for which the 22G-RNAs were depleted. Instead, we observed little change in the steady-

state levels of transcripts with depleted 22G-RNAs, indicating that NHL-2 is involved in the translation of these genes, or that there is a different role for NHL-2 with regard to these genes in the 22G-RNA pathways.

The lack of correlation between mRNAs with altered levels and changes in 22G-RNAs raises another possibility: that NHL-2 is mainly involved mainly in the biogenesis of a subset of the 22G-RNAs. This model seems plausible for several reasons. First, DRH-3 and NHL-2 physically interact by co-IP. Second, our metagene analysis of the distribution of 22G-RNAs along the length of target mRNA transcripts is similar to the pattern observed for *drh-3(ne4253)* mutants, in which the 22G-RNAs are reduced along the length of the gene body, with most significant decreases present at the 5' end of the transcript. This pattern is consistent with a role for NHL-2 in the processivity or activity of the RdRP complex on a subset of CSR-1 targets (Fig. 8). The RNA binding activity of NHL-2 points to a model whereby NHL-2 could help to identify particular mRNAs as candidates for 22G-RNA synthesis. Furthermore, because NHL-2 also associates with CSR-1 and HRDE-1 as well as DRH-3, it could also act as a chaperone required efficient handoff of 22G-RNAs from the RdRP complex to the Argonaute (Fig. 8).

This potential role for NHL-2 with the RdRP complex is noteworthy for several reasons. First, because TRIM/NHL proteins have thus far only been implicated in the effector step of miRNA pathways, this is the first indication that NHL-2 (and thus TRIM/NHL proteins) could also be involved in the biogenesis of endo-siRNAs. Second, we still have relatively little insight into the factors that route particular transcripts into the 22G-RNA pathways, and NHL-2 provides an attractive candidate for one such factor. Third, the role for NHL-2 in biogenesis of a subset of germline 22G-RNAs and the effector steps of somatic miRNAs demonstrate differential roles for this intriguing protein in the

germline versus soma, and points to differences in protein binding partners in each of these tissues that should be examined further.

Notably, many of the up-regulated genes in *nhl-2(ok818)* mutants were the targets of 22G-RNAs, yet these genes did not display alterations in the levels of 22G-RNAs. These data point to a role for NHL-2 in regulating transcript stability, in cooperation with the WAGOs, and in opposition to CSR-1, and suggest a combinatorial regulatory mechanism that engages both small RNA pathways and *bona fide* RBPs such as NHL-2. The fact that *nhl-2(ok818)* mutants display temperature dependent fertility defects is consistent with several small RNA pathway factors, including both the piRNA/WAGO and the CSR-1 pathways and points to NHL-2 as a co-factor and/or co-regulator required for optimal pathway activity under stressful conditions. Although NHL-2 was not identified previously as a factor in the piRNA or 22G-RNA pathways, our results exemplify the power of synthetic genetic screens to identify accessory factors involved in the optimal function these pathways.

In light of our data, we propose that NHL-2 acts as a hub of gene regulation, where it works cooperatively with core factors in a diverse set of pathways that are central to both somatic and germline gene regulation (Fig.8). NHL-2 localizes to several ribonucleoprotein structures involved in RNA regulation, including P granules in the germline, CGH-1 granules in the gonad core, and cytoplasmic P bodies in the soma, placing it in key cellular positions to regulate multiple facets of gene expression and RNA metabolism throughout development. For instance, P granules and related Mutator Foci (to which NHL-2 does not appear to localize) are thought to be important sites for the synthesis of 22G-RNAs and 22G-RNA-mediated gene regulation, as RdRP components and Argonautes, including CSR-1, WAGO-1, and HRDE-1 also localize to these sites (Phillips, et al., 2012; Claycomb, et al., 2009). CGH-1 granules of the gonad core have a complex interplay with P granules and are enriched

for translational regulators, including CGH-1 and IFET-1. The localization of NHL-2 to both P and CGH-1 granules in the germline supports the observation that NHL-2 and CGH-1 regulate the stability of a shared set of germline/oogenesis transcripts, and opens the possibility that NHL-2 and CGH-1 could regulate the translation of a shared set of targets. The intrinsic RNA binding capacity of NHL-2 via its NHL domain also makes it plausible that NHL-2 could regulate its own set of target mRNAs, independent of small RNA pathways or CGH-1. In fact, we observed that a large number of non-gonadal transcripts are regulated, both positively and negatively, in an NHL-2-dependent manner, and these transcripts are a separate group from those targeted by 22G-RNAs or CGH-1. Ultimately, quantitative proteomic studies, ribosome profiling in *nhl-2(ok818)* mutants, and NHL-2 RNA-IP or CLIP-seq experiments will enable us to identify the full repertoire of mechanisms of NHL-2 regulation.

610

611 CONCLUSIONS

In conclusion, we characterized the roles of NHL-2 in the germline and showed it localizes to P granules and impacts a subset of 22G-RNAs in both the CSR-1 and WAGO/HRDE pathways. This germline role in small RNA biogenesis is distinct from its role in the miRNA pathway in the soma, and implicates NHL-2 in RdRP activity in the germline. Interestingly, also NHL-2 was required for the nuclear RNAi pathway, suggesting that NHL-2 is a promiscuous co-factor of multiple distinct, but related small RNA pathways. NHL-2 displays intrinsic RNA binding ability via its NHL domain and thus is capable of binding and regulating the stability or translation of a large number of germline transcripts in a small RNA-independent manner. NHL-2 may exemplify a new class of co-factor that is required for optimal activity of small RNA pathways (both miRNA and 22G-RNA pathways). Although this type of co-factor appears to be extremely important for the fidelity and robustness of

developmental gene expression programs, additional examples of such co-factors are not likely to be identified by forward genetic screens, but may be revealed through similar screens in sensitized backgrounds. Overall, our study highlights the complex roles of the TRIM-NHL protein NHL-2 and lays the foundation for deeper mechanistic insights into how these versatile and conserved proteins act to regulate gene expression in various tissues.

EXPERIMENTAL PROCEDURES

Worm Strains

Strains used in this study were Bristol N2 as the wild-type, PRB310 (*nhl-2(ok818)*), YY186 (*nrde-2(gg091)*) and WM49 (*rde-4(ne301)*). Some strains were obtained from the *C. elegans* Genetics Centre (CGC, USA) and cultured under standard conditions (Brenner, 1974).

RNAi

RNAi was performed using the feeding method (Timmons and Fire, 1998). Each bacterial feeding clone was grown overnight in 2xTY media with 100µg/ml ampicillin and seeded onto NGM plates with 100µg/ml ampicillin and 4mM IPTG. Approximately 10 synchronized L1 animals of each strain were then pipetted onto each plate.

Brood Size Assay, Transgenerational and Mortal germline assays

Brood size assays were performed on animals fed *E. coli* OP50 or bacteria expressing RNAi clones. Synchronized populations of each strain were grown at 20°C and 25°C until the fourth larval stage (L4) and then individual L4 animals were then placed onto pre-seeded NGM plates and returned to their

respective temperatures. Animals were then transferred to new plates every 12 hours and plates were scored for progeny after 48 hours. This process was repeated until animals failed to lay new progeny. Total progeny included viable progeny and unhatched embryos, while embryonic lethality was scored separately as unhatched embryos. For transgenerational brood size analysis and Mrt assays, wild-type and homozygote *nhl-2(ok818)* worms were grown at 20°C for at least 5 generations and then shifted to 25°C as L1. In the transgenerational assay, F1 and subsequent generations were grown at either 25°C or 20°C for successive generations and total brood size and embryonic lethality scored. For the Mrt assay animals were maintained at 25°C and six L2/3 animals were transferred at each generation to individual plates and counting all progeny.

Nuclear RNAi assay

The *lir-1* RNAi clone was obtained from the ORFome RNAi library and sequence to confirm the identity. Synchronised L1 worms were plated on *lir-1* or control RNAi plates and grown at 25°C for 72 h and then inspected for arrested or dead worms.

Gonad Dissection and Immunostaining

One-day-old adults were anesthetized (0.01% tetramisole) and gonads dissected, snap frozen in liquid nitrogen and processed as described in (Navarro et al., 2001). Rabbit anti-NHL-2 antibodies was raised to the peptide RHESPATSTNNTQNS (GL Biochem, China). Anti-NHL-2 and anti-CGH-1 primary antibodies were incubated overnight at 4°C and secondary antibodies for 2 hours at 20°C. Slides were washed twice in PBS/ 0.1% Tween-20 for 10 minutes at 20°C and mounted for imaging using Dako Fluorescent Mounting Media (Dako, Denmark). Fluorescent images were acquired using an inverted

666 Olympus IX81 x 2-UCB microscope (Olympus, Tokyo, Japan) attached to an X-cite series 120Q
667 fluorescent light box.

668

669 **Immunoprecipitation and GST Pull-down Assay**

670 Full-length NHL-2 coding sequence was cloned into modified pDEST-Magic vector pTH6838,
671 resulting in an N-terminally-tagged NHL-2 expression construct. *Escherichia coli* C41 cells (Lucigen)
672 were transformed using NHL-2 expression construct or empty plasmid for tag-only control
673 experiments. Protein expression was induced by adding IPTG (1mM final) to overnight log phase cell
674 culture at 37°C, and continued for 3.5-4 hours. Cell lysates were prepared by sonication, and the added
675 to GST resin for binding. After washing off non-specific binders, tagged NHL-2 or GST protein was
676 eluted off the resin using 250 mM NaCl, 50 mM Tris-HCl (pH 8.8), 30 mM reduced glutathione, 10
677 mM BME and 20% Glycerol. Protein concentration and purity were estimated by SDS-PAGE using
678 standard procedure. Details for NHL-2 expression and purification processes can be found elsewhere
679 (Ray, et al., 2017; Ray, et al., 2013).

680

681 *C. elegans* lysate was prepared from gravid wild-type adults in 100 mM NaCl, 50 mM Sodium
682 phosphate (pH 7.5), 0.05% triton and a combination of protease (Roche) and phosphatase inhibitors.
683 For each binding experiment, 5 mg of worm lysate was pre-cleared using 25 ul of GST resin. 8 ug of
684 GST-tagged NHL-2 or 4 ug of GST protein was added to pre-cleared lysate containing fresh 25 ul of
685 GST resin in 1 ml final reaction volume. The mixture was incubated at 4°C for 3 hours. Proteins
686 unbound to the resin were removed by washing the resin 5 times with 50 ul 100 mM NaCl, 50 mM
687 sodium phosphate, 0.05% triton buffer. Bound proteins were eluted by adding 50 ul of 1X SDS gel

loading buffer to the resin followed by heating the samples at 70°C for 1 min. Candidate NHL-2 binding partners were separated on SDS- PAGE and probed by western blots using published *C. elegans* antibodies. Tubulin was used for loading controls between GST-NHL-2 and tag only experiments.

692

693 **Protein Expression, RNAcomplete and Fluorescence Anisotropy**

RNA pool generation, RNAcomplete pulldown assays and microarray hybridizations were performed as previously described (Ray, et al., 2017; Ray, et al., 2013; Ray, et al., 2009). Briefly, NHL domain of NHL-2 (residues 625-1032) was expressed as a GST-tagged fusion protein using the pGEX vector (pTH6838) (GE Healthcare). GST-NHL-2 (20 pmoles) and RNA pool (1.5 nmoles) were incubated in 1 mL of Binding Buffer (20 mM HEPES pH 7.8, 80 mM KCl, 20 mM NaCl, 10% glycerol, 2 mM DTT, 0.1 µg/µL BSA) containing 20 µL glutathione sepharose 4B (GE Healthcare) beads (pre-washed 3 times in Binding Buffer) for 30 minutes at 4°C, and subsequently washed four times for two minutes with Binding Buffer at 4°C. One-sided Z-scores were calculated for the motifs as described previously (Ray, et al., 2013).

For fluorescence anisotropy assays, *Escherichia coli* BL21(DE3) expressing GST-NHL-2 (729-1032) were grown to an OD₆₀₀ = 0.6-0.8 at 37°C and induced with the addition of 0.5 mM IPTG for 16 hr at 23°C. Cells were resuspended in lysis buffer (100 mM Tris.Cl pH 7.0, 5 mM EDTA, 5 mM DTT supplemented with 1 x 'cOmplete' Protease Inhibitor Cocktail (Roche), passed through a French press 4 times at 16,000-18,000 psi. and the lysate clarified by centrifugation at 8,000 g for 30 minutes. The GST-NHL-2 fusion protein was purified using a 5 mL GStrap FF based on the manufactures specification (GE healthcare). Alterations to the protocol included washing the column 5 x volumes of

binding buffer (1 x PBS pH 7.3) containing an additional 2 M NaCl to remove any DNA and RNA bound to NHL-2, followed by 5 column volumes of binding buffer. To elute the bound protein, the column was washed with elution buffer (1x PBS, 10mM reduced glutathione pH 8.0) and eluted fractions run on a SDS-PAGE gel to determine which fractions GST-NHL-2 eluted in, these fractions were then pooled and concentrated to be then further purified using size exclusion on the HiLoad 16/60 Superdex 200. The NHL domain purity was >95% by SDS-PAGE and was quantitated by OD₂₈₀ using an extinction coefficient of 61935 M⁻¹cm⁻¹ (Pace et al., 1995).

To examine the RNA-binding ability of the NHL domain, a 12-point serial dilution (0-52.81 μM) of GST and GST-NHL-2 NHL domain was incubated with 1 nM 5'-Fluorescein labelled 17 mer poly-U single-stranded RNA (Dharmacon GE, USA) in assay buffer (50 mM NaCl, 20 mM NaPO₄, 2 mM MgCl₂, 1 mM DTT, 10% glycerol pH 7.4) for 15 min at room temperature in 96-well non-binding black plates (Greiner Bio-One). Fluorescence anisotropy was measured in triplicate using PHERAstar FS (BMG) with FP 488-520-520 nm filters. Data were corrected for anisotropy of RNA alone samples, and then fitted to a one-site binding model using the Equation, $A = (A_{\max} [L]) / (K_D + [L])$, where A is the corrected fluorescence anisotropy; $A_{\max}$ is maximum binding fluorescence anisotropy signal, [L] is the NHL concentration, and K_D is the dissociation equilibrium constant. $A_{\max}$ and K_D were used as fitting parameters and nonlinear regression was performed using SigmaPlot 13.0.

Small RNA Cloning and Data Analysis

Total RNA was collected from wild-type and *nhl-2(ok818)* mutants that were grown to gravid adulthood at either 20°C or 25°C on OP50 *E. coli*. We isolated small RNAs and prepared libraries for Illumina sequencing using a previously described cloning strategy (Tu, et al., 2015; Gu, et al., 2009).

The only minor modification to these protocols was that we used 0.1X the normal amount of Tobacco Acid Pyrophosphatase (enabling us to capture a greater proportion of miRNAs, while still sufficiently recovering 22G-RNAs). Small RNA analysis was conducted using custom Shell and Perl (5.10.0) scripts (Tu, et al., 2015). The reference genome of *C. elegans* and genomic annotations were downloaded from WormBase (Release WS230) (Yook, et al., 2012). The snoRNAs annotated in GenBank were also included in the category of non-coding RNAs (ncRNAs). The sequences of pre-miRNAs and mature miRNAs were fetched from miRBase (Release 19). After removing the barcodes from small RNA-seq reads for each sample, the insert size were extracted by allowing at most one mismatch in the first 6 nt of the 3' linker (CTGTAG). We first excluded the reads that could be aligned to the ncRNAs, and then aligned the remaining reads to the genome using Bowtie (Langmead, et al., 2009) without mismatches. Small RNA abundance were normalized to the sequencing depth (the sum of genome-mapping and junction-mapping reads but those known ncRNAs) as reads per million (rpm). We used the same lists of CSR-1 targets, WAGO-1 targets, etc. as in (Tu, et al., 2015), and the same criteria to define the genes depleted of 22G-RNAs in *nhl-2(ok818)* mutants, i.e., at least 2-fold depletion in mutants than wild-type and at least 10 rpm reads in wild-type. The significance of the overlap between gene sets was calculated by hypergeometric test. The miRNA targets were predicted using TargetScan (Lewis, et al., 2005).

mRNA-Seq

Total RNA was extracted from triplicate samples of wild-type and *nhl-2(ok818)* mutants that were grown to gravid adulthood at either 20°C or 25°C on OP50 *E. coli*. mRNA-seq libraries were generated and sequenced at the Donnelly Sequencing Centre (University of Toronto) using the Illumina Tru-Seq

754 Stranded mRNA Library Construction Kit and the HiSeq2500 sequencer. The mRNA-seq reads were
755 mapped to the reference genome by Tophat (Trapnell, et al., 2009) with default parameters. For each
756 gene, we counted the number of the 22G-RNA reads that are antisense to the corresponding gene
757 transcript. With the counts, edgeR was used to integrate the three replicates of *nhl-2* mutants versus the
758 three replicates of wild-type. Then, a gene is called to be depleted of 22G-RNAs in *nhl-2* mutants if the
759 corresponding FDR and fold-change (FC) by edgeR satisfy $FDR < 0.05$ and $FC < \frac{1}{2}$, and the gene has
760 at least 10 reads per million (rpm) antisense 22G-RNAs reads in at least one of three replicates of wild-
761 type.

762

763 **ACCESSION NUMBERS**

764 All small RNA and mRNA Illumina sequencing data have been submitted to the NCBI's Sequence
765 Read Archive (SRA), and are included under project accession number SRP115391.

766

767 **AUTHOR CONTRIBUTIONS**

768 GMD, JMC, and PRB conceived of and designed all experiments. GMD analyzed brood sizes,
769 performed germline imaging and phenotype quantification, characterized the NHL-2 antibody, and
770 performed NHL-2 co-IP experiments with assistance from JMTA. RNC, MJC, and JAW performed
771 and analyzed the anisotropy RNA-binding experiments. DR performed the RNACOMPETE assay. MAF
772 isolated small RNA and mRNA fractions for Illumina sequencing and cloned the small RNA Illumina
773 libraries. MZW performed DRH-3 co-IP experiments in Fig. S6. ST and ZW performed bioinformatics
774 analysis. JMC and PRB wrote the manuscript with input from all authors.

775

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786

787 SUPPLEMENTAL INFORMATION

788 Supplemental Information includes seven Supplemental Figures and three Supplemental Tables.

789 Figure S1. Comparison of representative TRIM-NHL proteins, Related to Figure 1.

790 Figure S2. The NHL-2 antibody is specific, Related to Figures 1, 2.

791 Figure S3. Synthetic brood size reduction and embryonic lethality between *nhl-2(ok818)* and the CSR-1
792 pathway, Related to Figure 2.

793 Figure S4. Length and first nucleotide distribution of small RNAs in wild-type and *nhl-2(ok818)*
794 mutants at 20°C.

795 Figure S5. Length and first nucleotide distribution of small RNAs in wild-type and *nhl-2(ok818)*
796 mutants at 25°C.

797 Figure S6. Comparison of abundance (in rpm) for different categories of small RNAs in wild-type and
798 *nhl-2(ok818)* mutants at 20°C vs. 25°C.

799 Figure S7. The distribution of 22G-RNAs is reduced at the '5-end of CSR-1 targets in *nhl-2(ok818)*
800 relative wild-type control at 25°C. Related to Figure 4.

801 Figure S8. The RdRP complex is intact in *nhl-2(ok818)* mutants, Related to Figures 2, 4.

802 Table S1: Small RNA sequencing statistics of four samples in *nhl-2(ok818)* mutants and wild-type
803 animals at 25°C or 20°C, Related to Figures 4, 6.

804 Table S2: Genes altered in small RNA and mRNA sequencing datasets generated and investigated in
805 this paper, Related to Figures 4, 6.

806 Table S3: Master table of small RNA and mRNA-seq data used in this study.

807

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977

Figure 1. NHL-2 is required for fertility and is enriched in germline granules. (A) Average brood size and (B) embryonic lethality of *nhl-2(ok818)* mutants compared to wild-type at 20°C, 23°C and 25°C. Embryonic lethality is significantly different to wild-type animals at higher temperatures but not significantly different to wild-type at 20°C. Brood size is significantly lower across 20°C, 23°C and 25°C when compared to wild-type animals. ***=P values <0.001, * error bars represent standard error of mean. (n=5) (C) Representative images of DAPI stained diakinetoc oocytes showing abnormal chromosome morphology in *nhl-2(ok818)* when compared to wild-type animals. *nhl-2(ok818)* chromosomes show aggregation or greater than six chromosomal bodies. Scale bar 5µm, n=20 (D) Quantification of chromosomal defects in diakinetoc oocytes in wild-type and *nhl-2(ok818)* animals at 25°C. (E) NHL-2 co-localizes with CGH-1 in P-granules (top panels, Surface) and gonadal core CGH-1 granules (bottom panels, Core). DAPI (blue), NHL-2 (red), CGH-1 (green). Scale bar 10µm. Immunostaining with affinity purified NHL-2 antibody was reduced to background in *nhl-2(ok818)* germlines (Fig. S2A).

Figure 2. NHL-2 genetically interacts with the CSR-1 22G-RNA pathway. (A) Representative images of DAPI stained oocyte diakinetoc chromosomes showing abnormal chromosome morphology when *drh-3*, *csr-1*, *ekl-1* and *cde-1* were knocked down by RNAi in wild-type and *nhl-2(ok818)* animals. Scale bar 5µm, n=90 oocytes. (B, C, D & E) Chromosomes from each phenotype were binned in one of four categories: normal, oocytes with >6 chromosomal bodies, aggregated chromosomes, and enhanced aggregation (where aggregation exceeded that observed in wild-type animals). (F) Pachytene region of dissected adult male germlines stained with DAPI (red) and H3K9me2 (green) in wild-type males treated with *drh-3(RNAi)*, *csr-1(RNAi)* and *nhl-2(ok818)* mutants. Negative control wild-type

male presented on the far left (WT XO) showing one strong signal of H3K9me2 (yellow arrowheads). *drh-3(RNAi)*, *csr-1(RNAi)* and *nhl-2(ok818)* mutants display abnormal accumulation or distribution of H3K9me2. Scale bar 50µm, n=30 germlines. **(G)** Quantification of H3K9me2 distribution in germ cells from each phenotype scored as normal, elevated, or dispersed from each phenotype. *nhl-2(ok818)* mutants display similar amounts of germ cells with abnormal H3K9me2 markings.

Figure 3. Analysis of nuclear RNAi pathways in *nhl-2(ok818)* mutants. **(A)** The nuclear RNAi pathway silence somatic targets. dsRNA targeting the *lir-1* mRNA leads to the generation of 22G small RNAs in the cytoplasm and the nuclear RNAi pathway uses these 22G RNAs to target the endogenous *lir-1/let-26* locus for silencing. **(B)** *nhl-2(ok818)* worms are resistant to *lir-1* RNAi. Percent larval arrest represents mean of two biological replicates $\pm$ SD; n> 100. **(C)** *nhl-2(ok818)* worms display a mortal germline phenotype at 25°C. Error bars indicate mean $\pm$ SD, n=6. **(D)** Analysis of transgenerational broad size and embryonic lethality **(E)** of wild-type and *nhl-2(ok818)* worms. ****=P values <0.0001, ***=P values <0.0004, *=P values <0.0418. Error bars indicate mean $\pm$ SD, n=37.

Figure 4. NHL-2 interacts physically with the components of the 22G-RNA pathway. **(A)** Western blot using anti-CSR-1 showing no variance between CSR-1 levels in wild-type animals (WT) and *nhl-2(ok818)* mutants when equal amounts of protein are loaded. Tubulin is shown as a loading control (bottom). CSR-1 co-immunoprecipitates with NHL-2. NHL-2 was immunoprecipitated from one-day-old adult hermaphrodite extracts and CSR-1 was detected by Western blot. **(B)** DRH-3 co-immunoprecipitates with NHL-2. NHL-2 was immunoprecipitated from one-day-old adult hermaphrodite extracts and DRH-3 was detected by Western blot. **(C)** GST-pulldown of NHL-2. GST-

NHL-2 was able to pulldown CSR-1 and HRDE-1, but not WAGO-1. SDS-PAGE gel of purified GST-NHL-2 is shown in Supplemental Figure S8B. **(D)** Western blot using anti-CSR-1 showing no variance between CSR-1 levels in wild-type animals (WT) and *nhl-2(ok818)* mutants when equal amounts of protein are loaded. Tubulin is shown as a loading control (bottom).

Figure 5. Comparison of small RNAs in *nhl-2(ok818)* mutants to other small RNA and mRNA profiles. **(A)** Venn-pie diagrams show comparisons between sets of genes that are the targets of 22G-RNAs. For mutant strains, genes are two-fold or greater depleted of 22G-RNAs in mutants relative to appropriate wild-type controls, with a minimum of ten rpm. For Argonaute IPs, genes are two-fold or greater enriched for 22G-RNAs in the IP relative to a total small RNA input sample, with a minimum of ten rpm. Numbers shown in bold demonstrate statistically significant overlap. **(B)** Venn-pie diagrams show comparisons between 22G-RNA gene targets (as in (A), left/rows), and mRNA transcriptome data (bottom/columns) from Total gonads (the union of entire male and female gonad transcriptomes), male gonads (Spermatogenesis), female gonads (Oogenesis), and gender neutral genes (Ortiz, et al., 2014)(the overlap between genes expressed in both male and female gonads). Numbers shown in bold demonstrate statistically significant overlap. **(C-E)** Distribution of 22G-RNA reads in *nhl-2(ok818)* mutants along the scaled gene bodies are shown for CSR-1 target genes (C), WAGO-1 target genes (D), and HRDE-1 target genes (D) in *nhl-2(ok818)* and wild-type animals.

Figure 6. The NHL domain of NHL-2 binds RNA. **(A)** Side and top view of the homology-based structure prediction of the NHL-2 NHL domain and its six β -propellers marked blue. Electrostatic surface representation of the NHL-2 NHL domain showing negative regions in blue and positive in red

1044 is shown below. The six blades of the β propeller of the NHL domain are numbered (I–VI). **(B)**
 1045 Identification of RNA-binding motif of purified GST-NHL-2-NHL domain by RNAcompete. The top
 1046 five high-scoring 7-mers, and the scatter plots, displaying Z scores and motifs for the two halves of the
 1047 RNA pool (set A and set B) are shown. Sequence logos were derived by aligning the top ten high-
 1048 scoring 7-mers. **(C)** Quantitative assessment of RNA-binding of GST-NHL-2 NHL domain and GST.
 1049 The fluorescence anisotropy of reactions contained 1 nM fluorescently-labeled 17mer poly-U single-
 1050 stranded RNA and increasing concentrations of GST-NHL-2 NHL domain (closed circles) or GST
 1051 alone (open circles).

1052

1053 **Figure 7. Analysis of the 22G-RNAs targeting the *nhl-2(ok818)* mRNA transcriptome. (A)** Venn-
 1054 pie diagrams indicate the number of genes enriched in or depleted of 22G-RNAs in *nhl-2(ok818)*
 1055 mutants relative to wild-type worms at 20°C and 25°C, as determined using EdgeR. Numbers in bold
 1056 demonstrate statistically significant overlap. Each row corresponds to a gene set, with its label on the
 1057 right. Each Venn-pie diagram indicates the overlap between the gene set of its row and the gene set
 1058 whose label is in its column. **(B)** Venn-pie diagrams show comparisons between the predicted targets of
 1059 the miRNAs *let-7* and *lsy-6* (determined using TargetScan6), and genes that are mis-regulated in *nhl-*
 1060 *2(ok818)* mutants. **(C)** Venn-pie diagrams show comparisons between the genes depleted of 22G-
 1061 RNAs in *nhl-2(ok818)* mutants and the genes enriched in 22G-RNAs in CSR-1, WAGO-1, or HRDE-1
 1062 IP samples with *nhl-2(ok818)* mRNA-seq data. **(D)** Venn-pie diagrams show comparisons between *nhl-*
 1063 *2(ok818)* mRNA-seq data and genes depleted of 22G-RNAs in *glp-4(bn2)* mutants (which have very
 1064 few germ cells) or germline expressed gene mRNA-seq data (Ortiz et al., 2014) (as in Figure 4B).

1065

1066 **Figure 8. Model: NHL-2 acts as a hub of gene regulatory activity. (A)** NHL-2 interacts with the
 1067 miRNA pathway in the soma, where it may regulate the translation of *let-7* and *lsy-6* targets. The NHL
 1068 RNA binding domain of NHL-2 may help to reinforce target selection of miRISC in this pathway. **(B)**
 1069 In the germline, NHL-2 interacts with the 22G-RNA pathway, specifically via genetic and physical
 1070 interactions with the RdRP helicase DRH-3. In this capacity, NHL-2 could impact the processivity or
 1071 reloading of the RdRP complex on mRNA templates, and/or could aid in target mRNA selection via its
 1072 RNA binding capacity. Owing to its genetic and physical interaction with CSR-1, NHL-2 could also be
 1073 involved in the handoff of 22G-RNAs from the RdRP complex to Argonaute. If NHL-2 mediates gene
 1074 regulation via 22G-RNAs, it is likely to be at the level of translation. **(C)** The intrinsic RNA binding
 1075 capacity of NHL-2 could enable it to regulate a large number of transcripts in the soma and germline,
 1076 both positively and negatively. This activity could be influenced by additional binding partners, and we
 1077 speculate that it may occur in P bodies (soma) and P granules (germline) based on the localization
 1078 pattern of NHL-2.

1079 Supplemental figures

1080 Figure S1. Comparison of representative TRIM-NHL proteins.

1081 (A) Domain organization of selected TRIM-NHL family proteins. The TRIM-NHL family of proteins
1082 are conserved among metazoans, however, they often display variable domain organization. Canonical
1083 TRIM-NHL proteins have a three zinc-binding domains (a RING finger, one or two B-box motifs) and
1084 a coiled-coil domain in the N-terminal half of the proteins, and a series of NHL repeats at the C-
1085 terminal end of the protein. Examples shown are from: *Drosophila melanogaster* (BRAT and Mei-
1086 P26), *C. elegans* (NCL-1 and NHL-2) and *Homo sapiens* TRIM32.

1087

1088 Figure S2. The NHL-2 antibody is specific.

1089 (A) NHL-2 and CGH-1 staining of dissected *nhl-2(ok818)* hermaphrodite germlines demonstrates that
1090 the NHL-2 antibody is specific and that CGH-1 localizes properly in the absence of *nhl-2*. Scale bar 50
1091 μ m. (B) NHL-2 and CGH-1 co-staining of wild-type embryos show that NHL-2 is enriched in the germ
1092 cell lineage (P lineage, posterior/right), where it is found in the cytoplasm and in P granules
1093 (arrowheads). Scale bar 10 μ m. (C) NHL-2 and CAR-1 co-staining of temperature sensitive mutant
1094 *cgh-1(tn691)* grown at the restrictive temperature shows that NHL-2 does not co-localize with CAR-1
1095 in the solid sheet in the gonad core. Arrowheads point to the CAR-1 solid sheets. Scale bar 10 μ m.

1096

1097 Figure S3. Synthetic brood size reduction and embryonic lethality between *nhl-2(ok818)* and the

1098 CSR-1 pathway. (A) Knockdown of CSR-1 pathway genes in *nhl-2(ok818)* mutants results in
1099 synthetically reductions in brood size that are significantly different than knockdown in wild-type
1100 animals. (B) Average embryonic lethality of unhatched embryos from the same brood size assay.

Knockdown of CSR-1 pathway genes in *nhl-2(ok818)* mutants results in significantly higher embryonic lethality compared to knockdown in wild-type animals. ***=P values<0.001, error bars represent standard error of mean. *n*=5.

1104

Figure S4. The RdRP complex is intact in *nhl-2(ok818)* mutants. (A) Western blot using anti-CSR-1 showing no variance between CSR-1 levels in wild-type animals (WT) and *nhl-2(ok818)* mutants when equal amounts of protein are loaded. Tubulin is shown as a loading control (bottom) (B) SDS-PAGE gel of purified GST-NHL-2 and GST used in GST Pull-down experiments. Arrow heads indicate the positions of GST-NHL-2 and GST (C) An IP of DRH-3 also precipitates the RdRP complex components EKL-1 and EGO-1 in wild-type and *nhl-2(ok818)* mutants at 25°C. Western blots are probed with anti-EGO-1 (top), anti-DRH-3 (center), anti-EKL-1 (bottom). Sizes are marked, and a mock IP (beads only) is shown as a negative control. All antibodies are as used in (Claycomb et al., 2009, Gu et al., 2009). (D) A darker exposure of the DRH-3 and mock IP lanes.

1114

1115

Figure S5. Analysis of small RNA populations in *nhl-2(ok818)* mutants and wild-type animals at 20°C. (A) Length and first nucleotide distribution of small RNAs in wild-type and *nhl-2(ok818)* mutants at 20°C. Small RNA abundance is measured in reads per million (rpm) for all small RNAs that are mapped to the genome or exon-exon junctions. (B) Proportions of reads which were aligned to 21U-RNAs (piRNAs), miRNAs, sense (S) mRNAs, antisense (AS) mRNAs, pseudogenes, RepeatMasker annotated repeat elements (RMSK), and the unannotated portion of the genome, with

the first nucleotide distribution of small RNAs in each category (Note that all classes except for miRNAs and piRNAs include 22G-RNAs and 26G-RNAs).

1124

Figure S6. Analysis of small RNA populations in *nhl-2(ok818)* mutants and wild-type animals at 25°C. (A) Length and first nucleotide distribution of small RNAs in wild-type and *nhl-2(ok818)* mutants at 25°C. Small RNA abundance is measured in reads per million (rpm) for all small RNAs that are mapped to the genome or exon-exon junctions. **(B)** Proportions of reads which were aligned to 21U-RNAs (piRNAs), miRNAs, sense (S) mRNAs, antisense (AS) mRNAs, pseudogenes, RepeatMasker annotated repeat elements, and the unannotated portion of the genome, with the first nucleotide distribution of small RNAs in each category (Note that all classes except for miRNAs and piRNAs include 22G-RNAs and 26G-RNAs).

1133

Figure S7. Comparison of abundance (in ppm) for different categories of small RNAs in wild-type (green) and *nhl-2(ok818)* mutants (blue) at 20°C vs. 25°C. Biological replicates are indicated as 1, 2 and 3.

1137

Figure S8. The distribution of 22G-RNAs is reduced at the 5'-end of CSR-1 targets in *nhl-2(ok818)* relative wild-type control. Along the scaled gene body, the coordinates of the centroids of the areas under the 22G-RNA density curves (Fig. 4C-E) were calculated and compared. The centroid positions for 4,392 CSR-1 target genes between mutants and wild-type controls were compared in boxplot with Wilcoxon rank sum test p-values (A) and scatter plots (B,C) with t-test p-values.

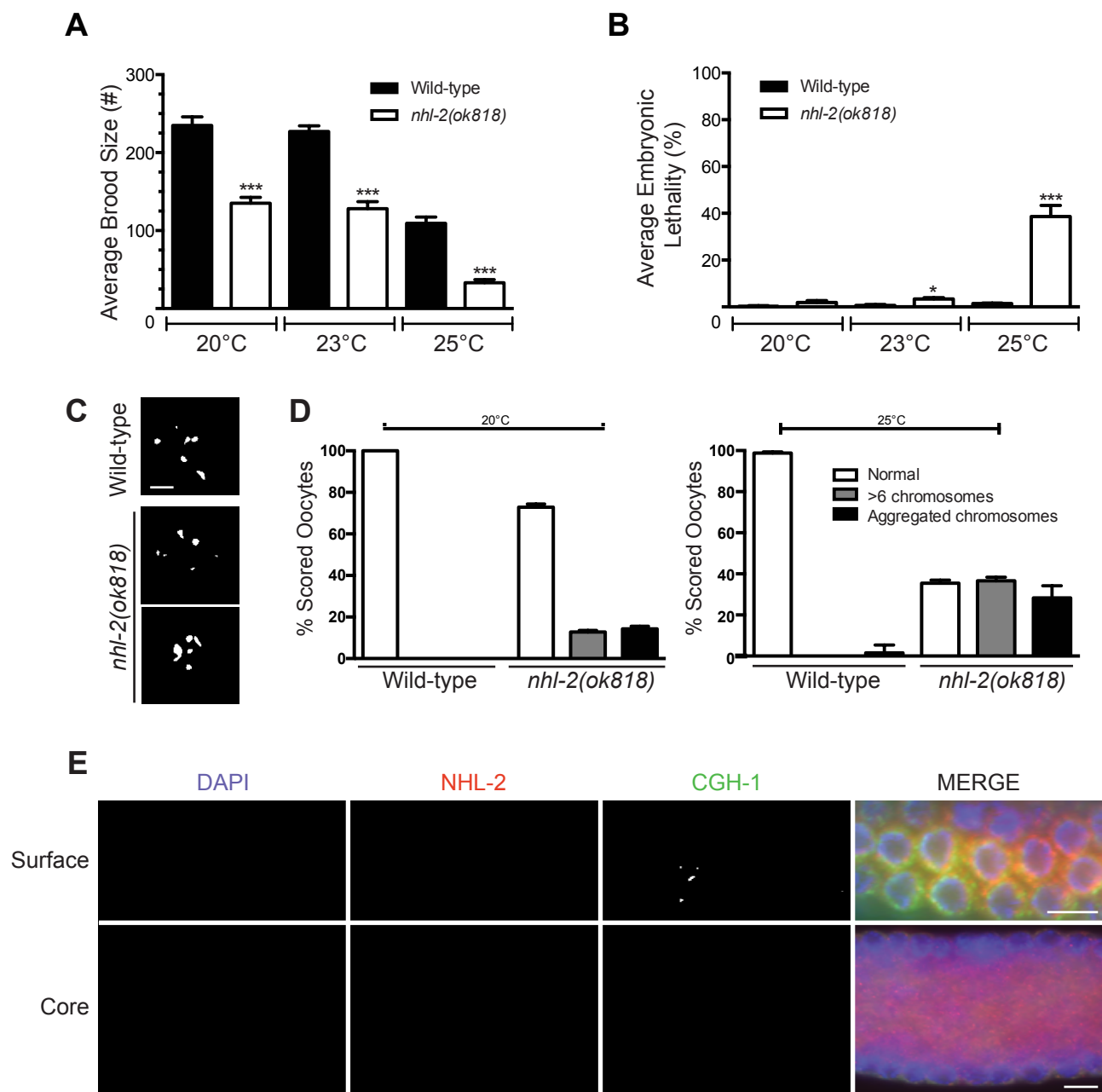
1143

1144 **Supplemental Tables**

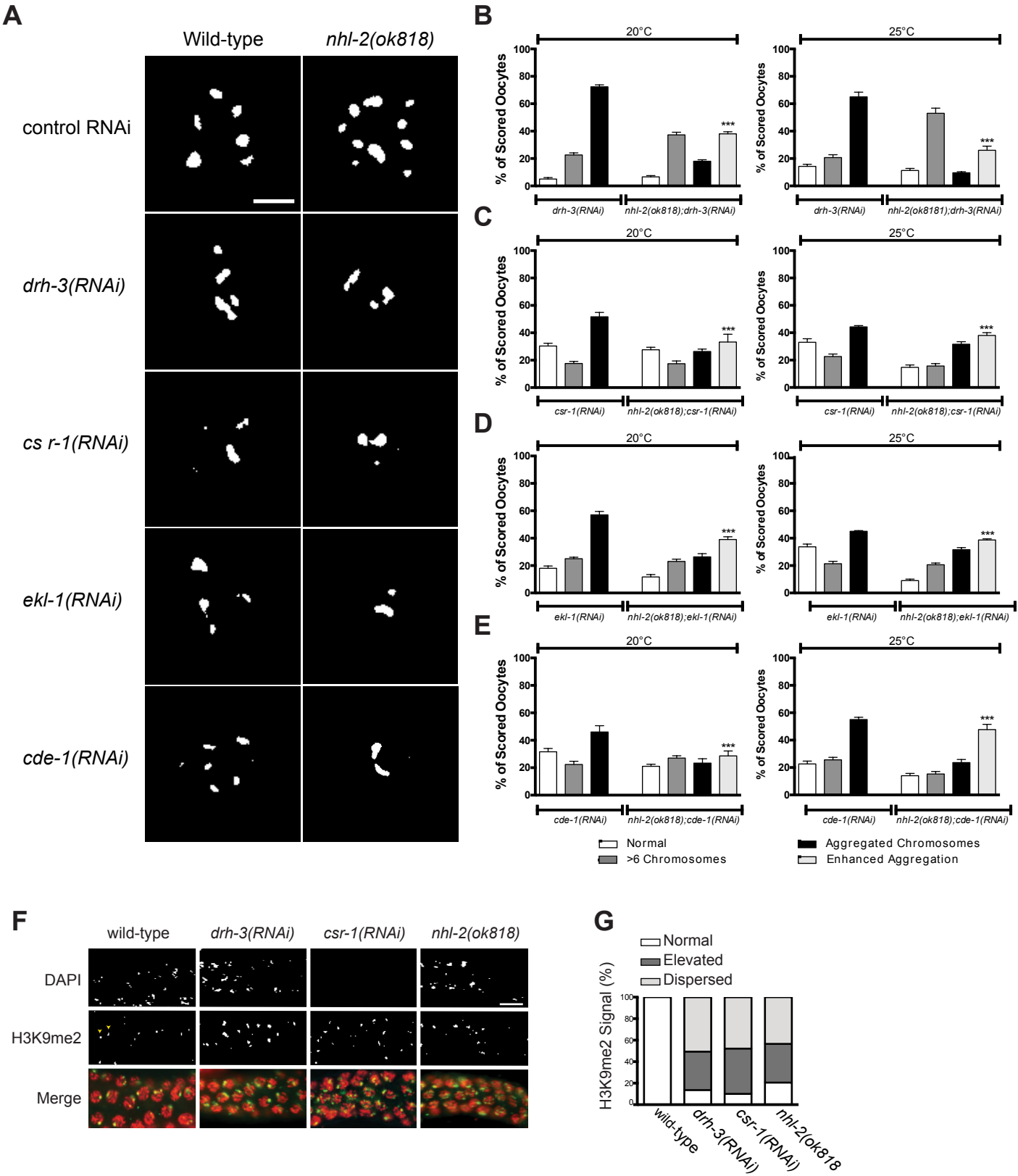
1145 Table S1: Small RNA sequencing statistics of four samples in *nhl-2(ok818)* mutants and wild-type
1146 animals at 25°C or 20°C.

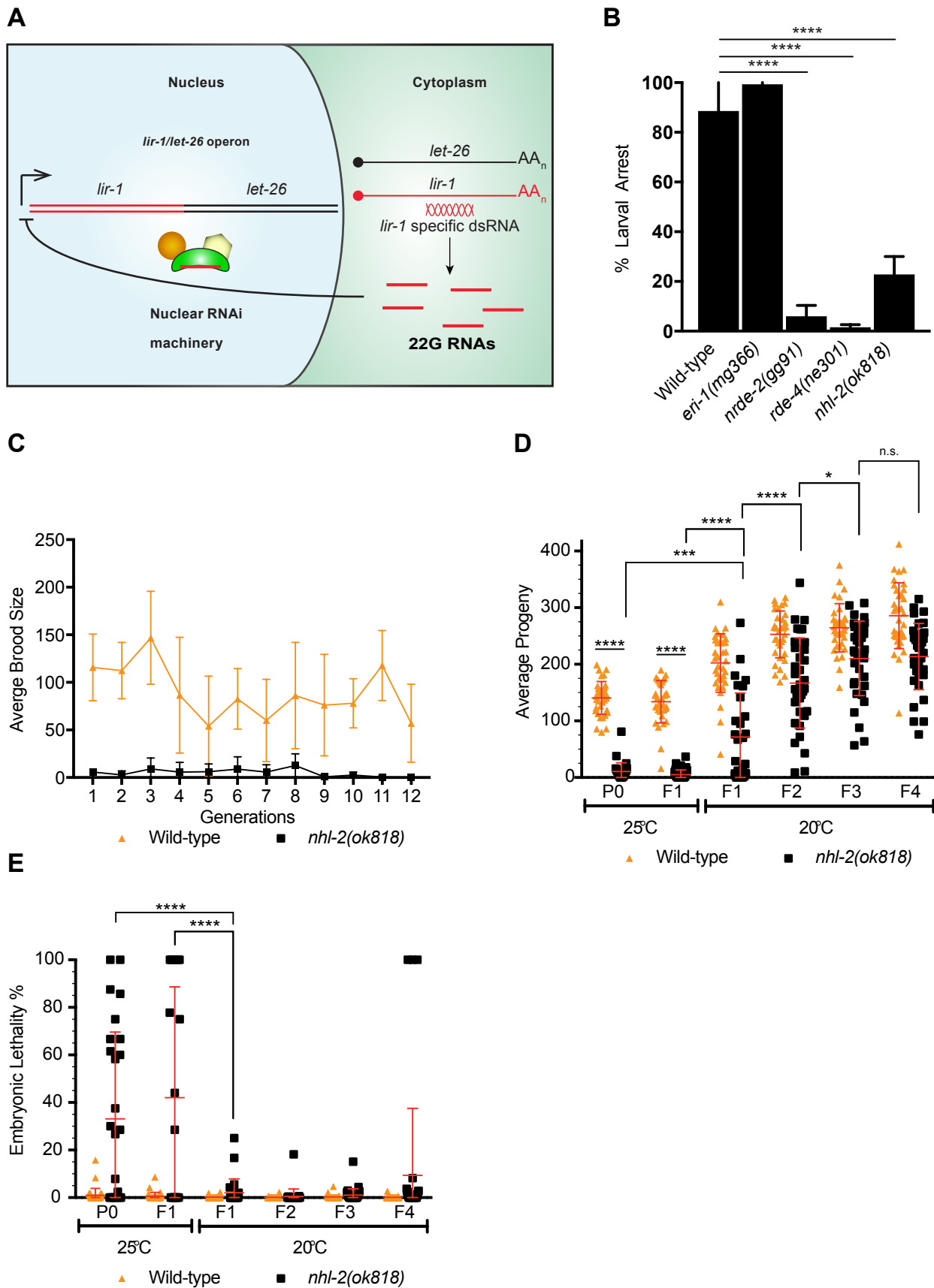
1147 Table S2: Genes altered in small RNA sequencing datasets generated and investigated in this paper.

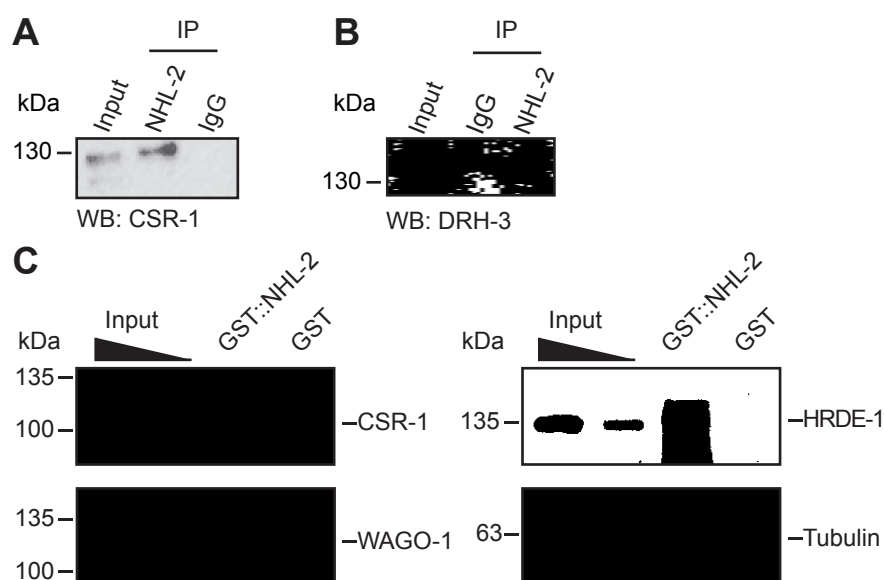
1148 Table S3: Master table of small RNA and mRNA-seq data used in this study.

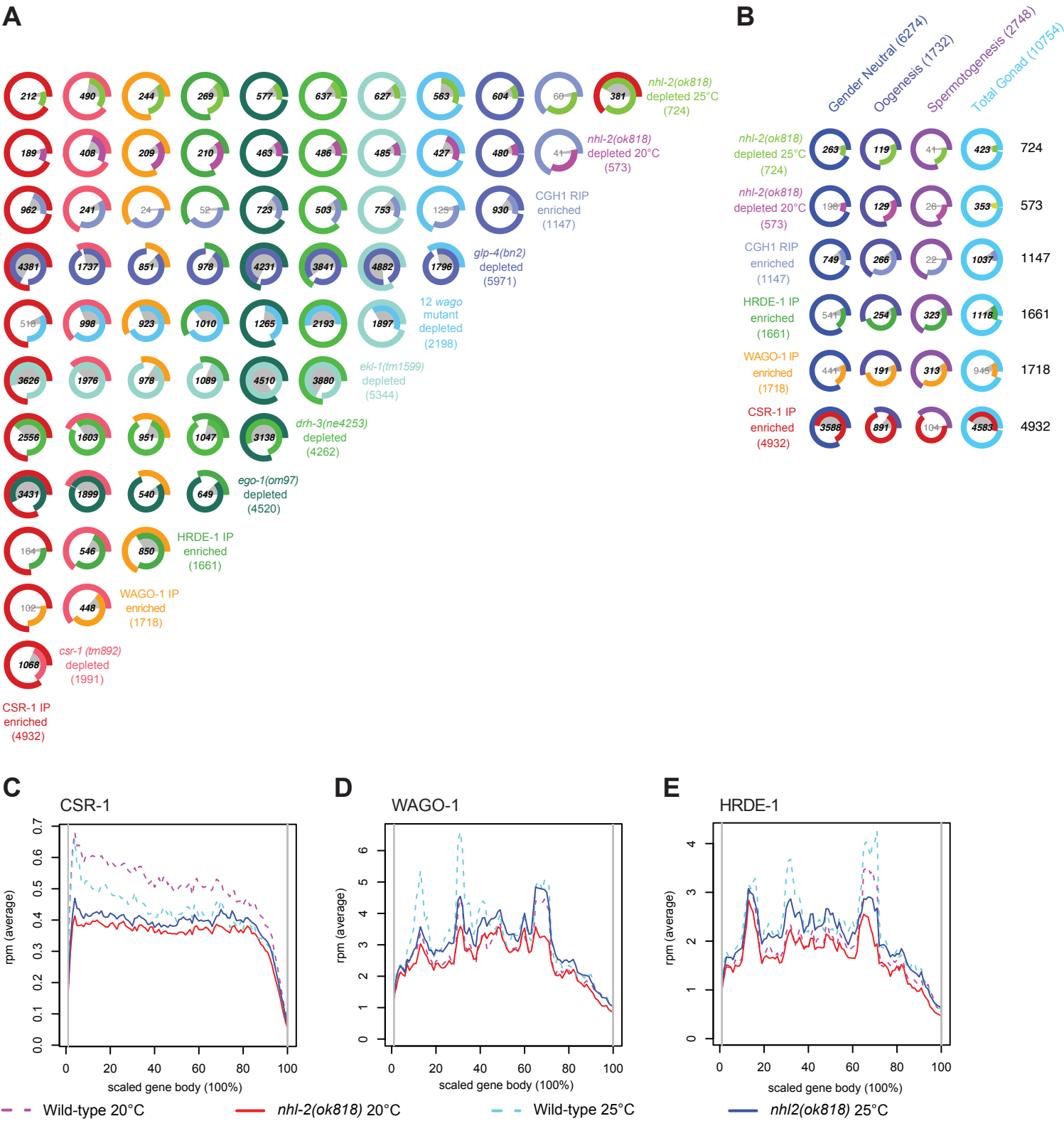


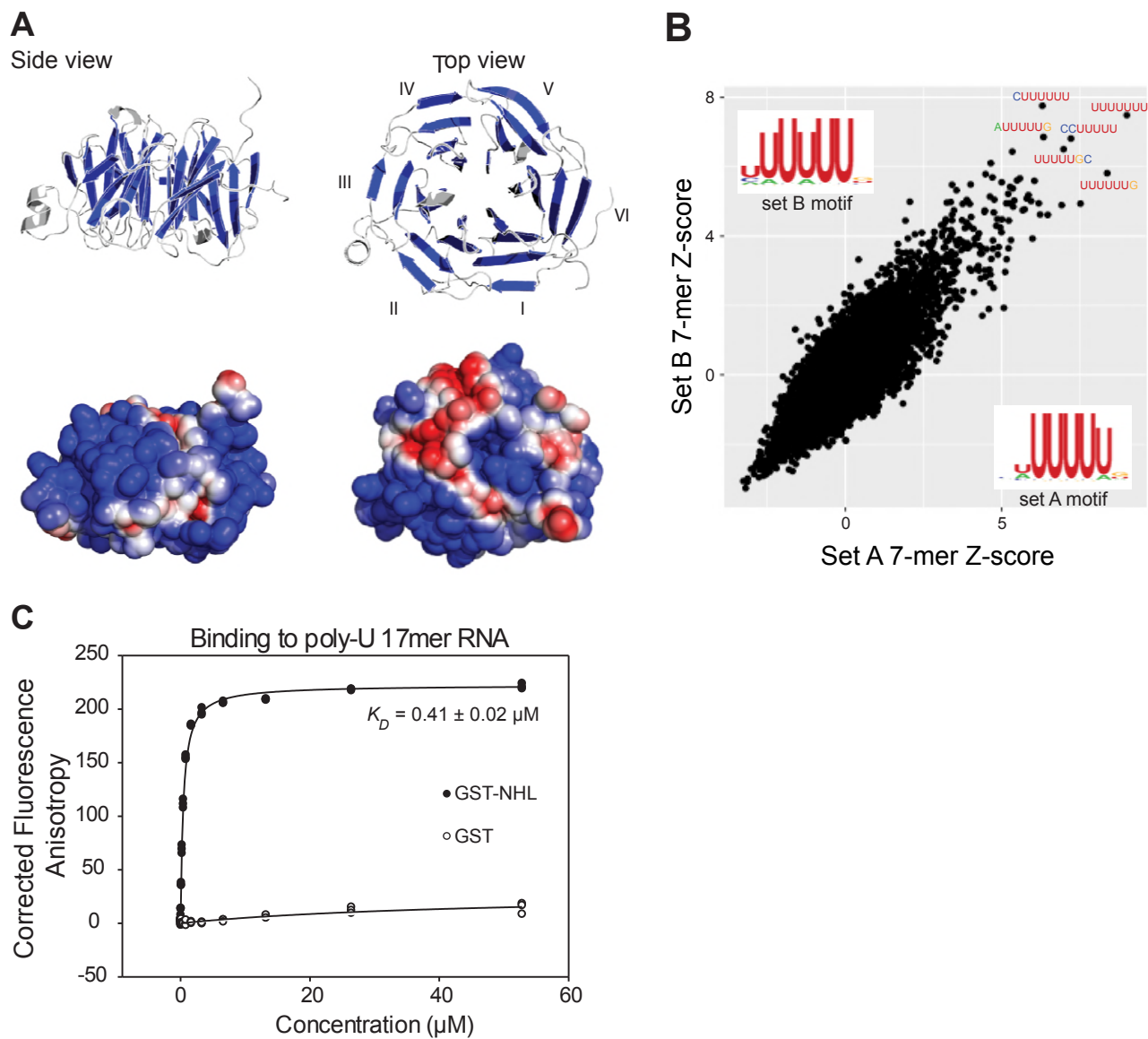
Davis et al Fig. 2

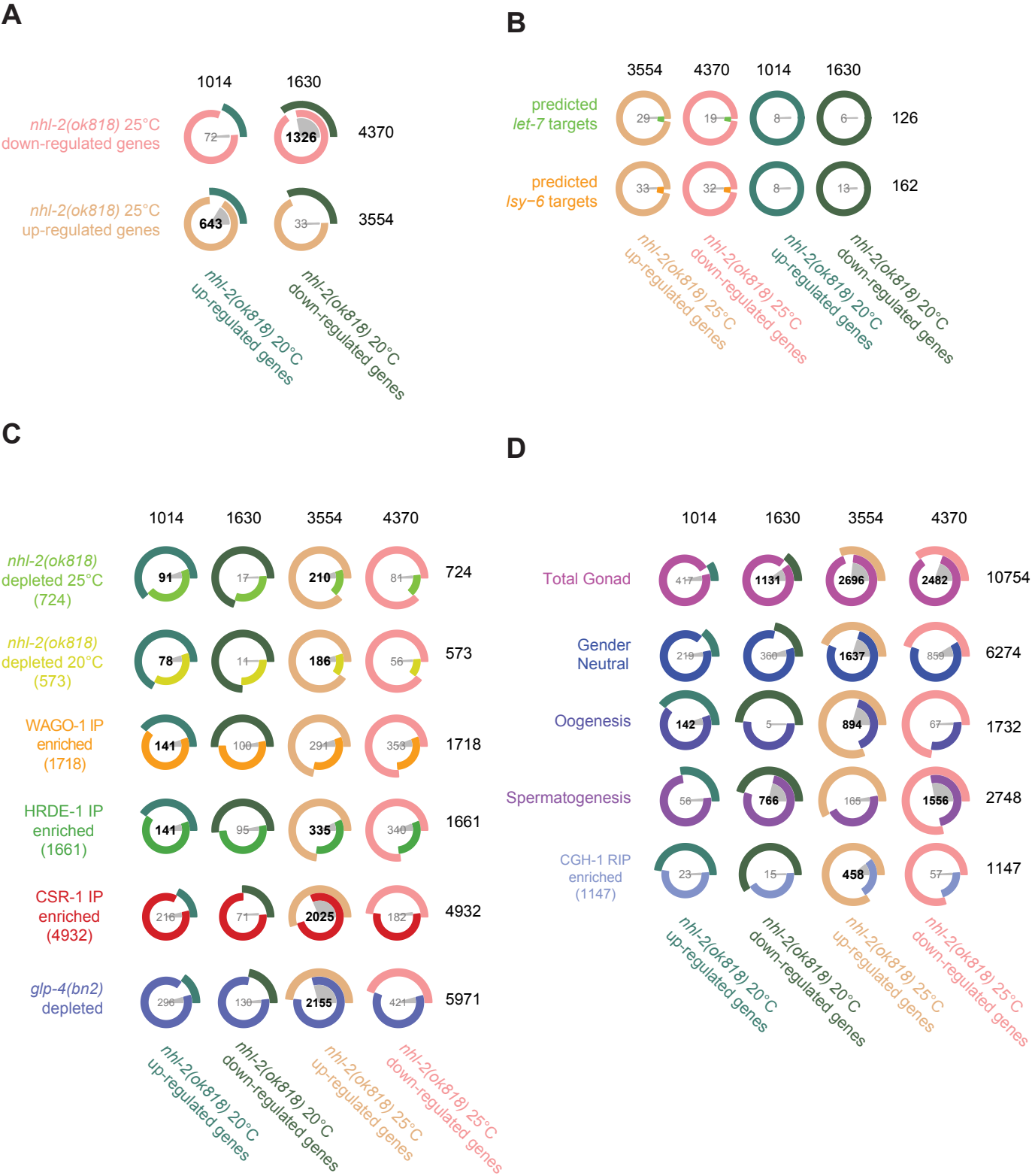


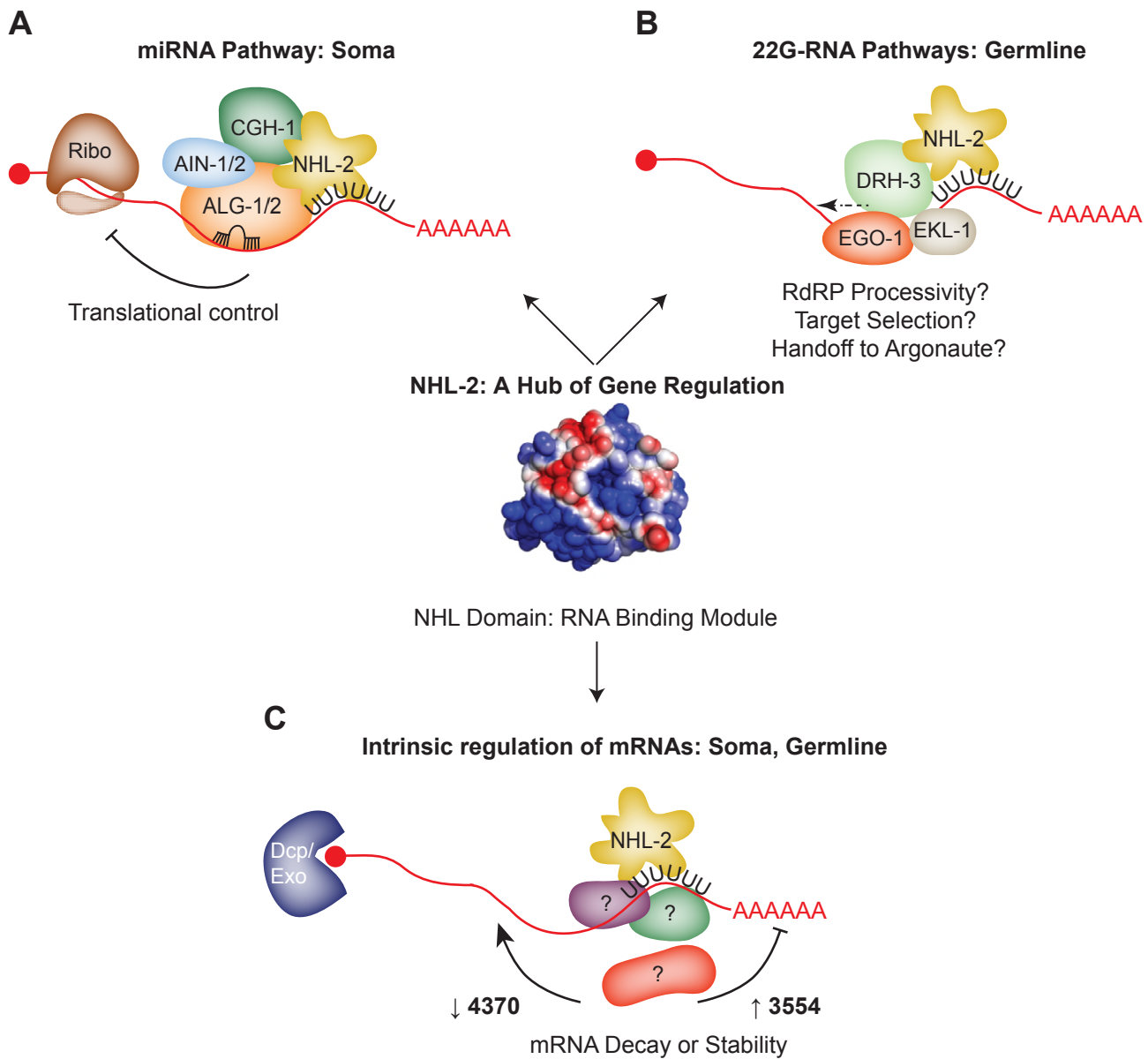




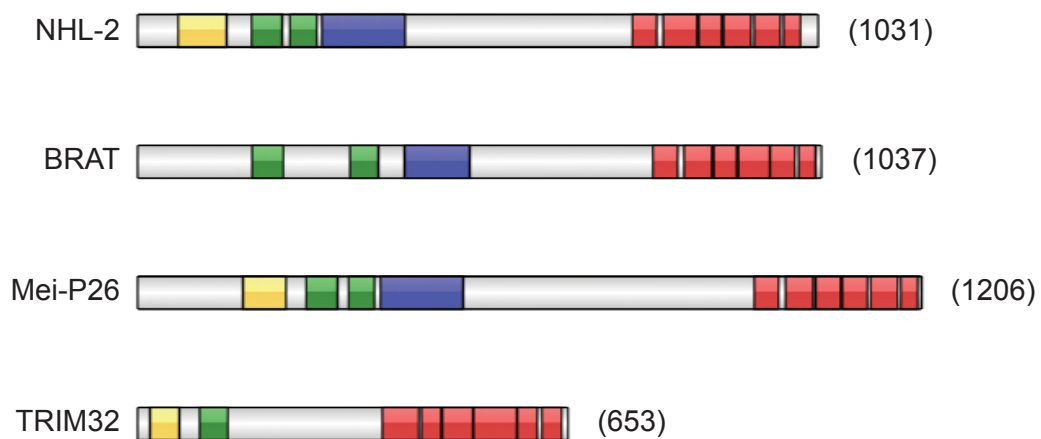






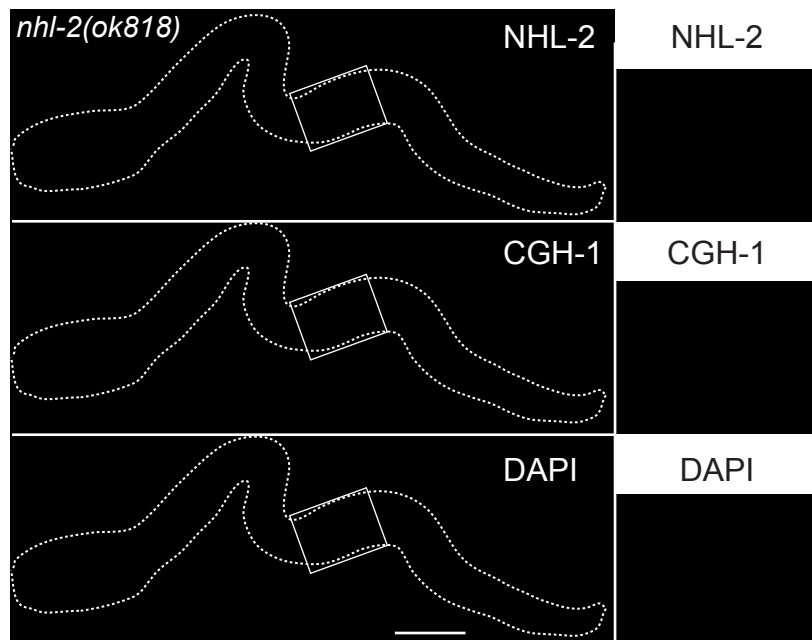


Davis Supplemental Fig. S1



Davis Supplemental Fig. S2

A



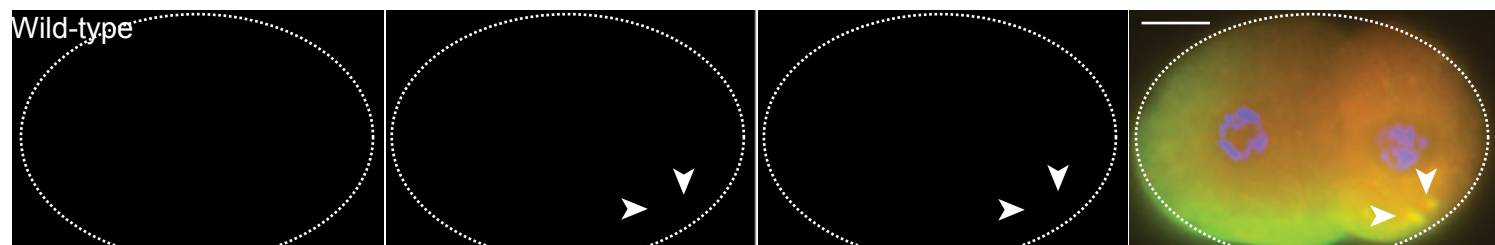
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CGH-1

MERGE



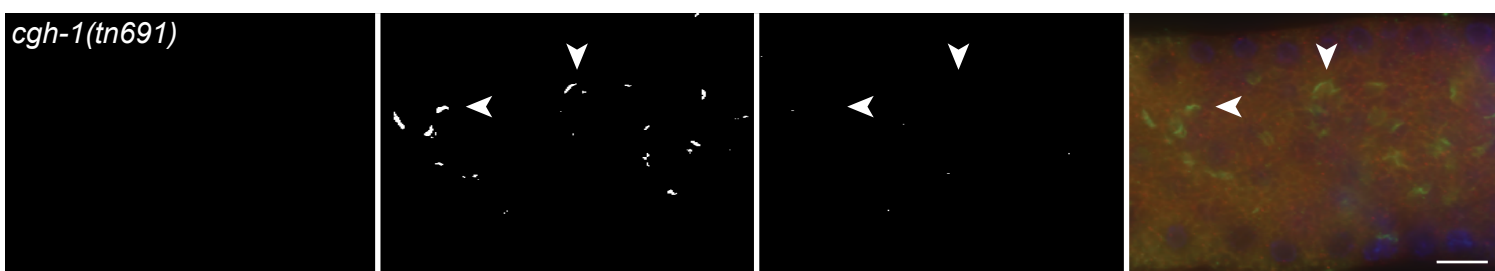
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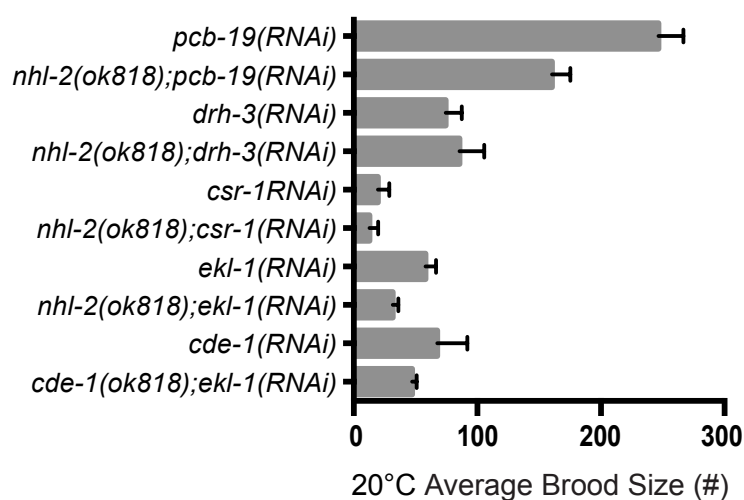
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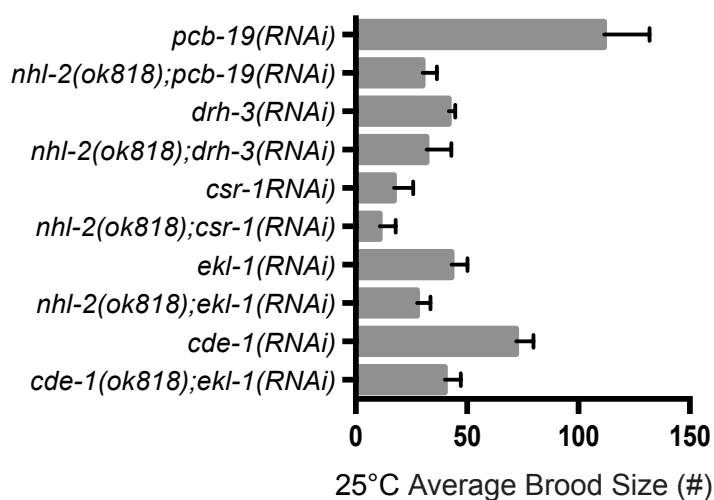
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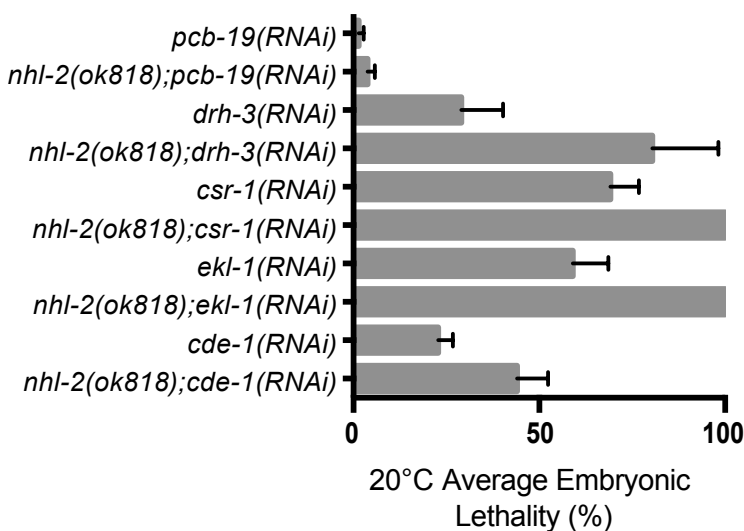
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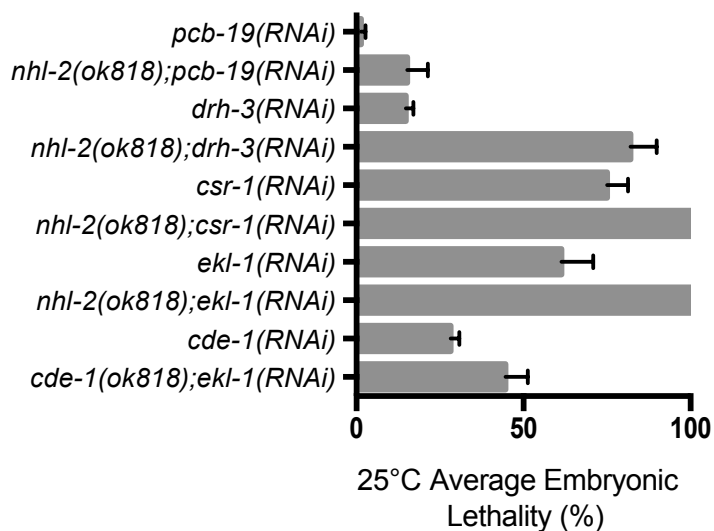
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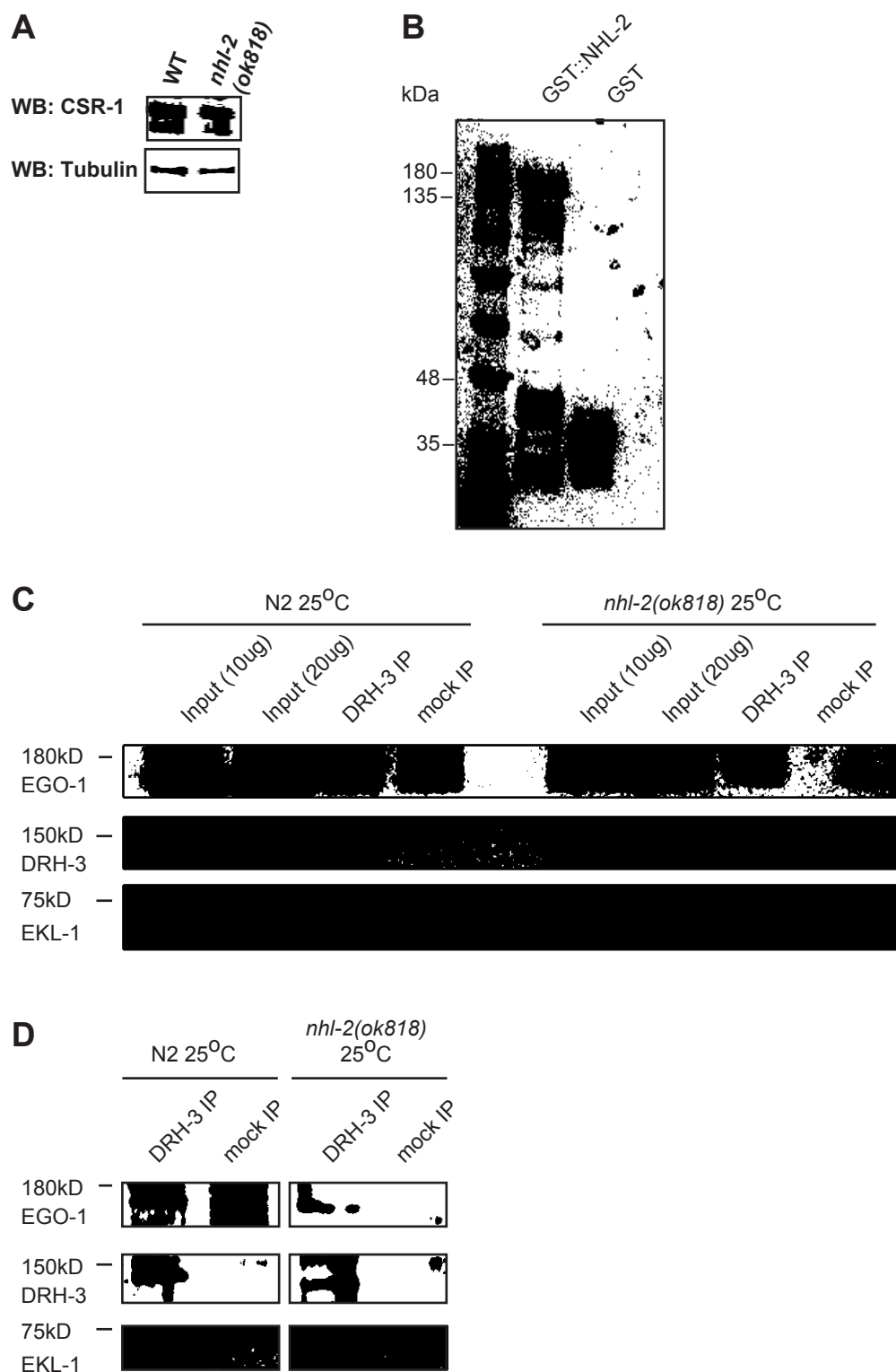


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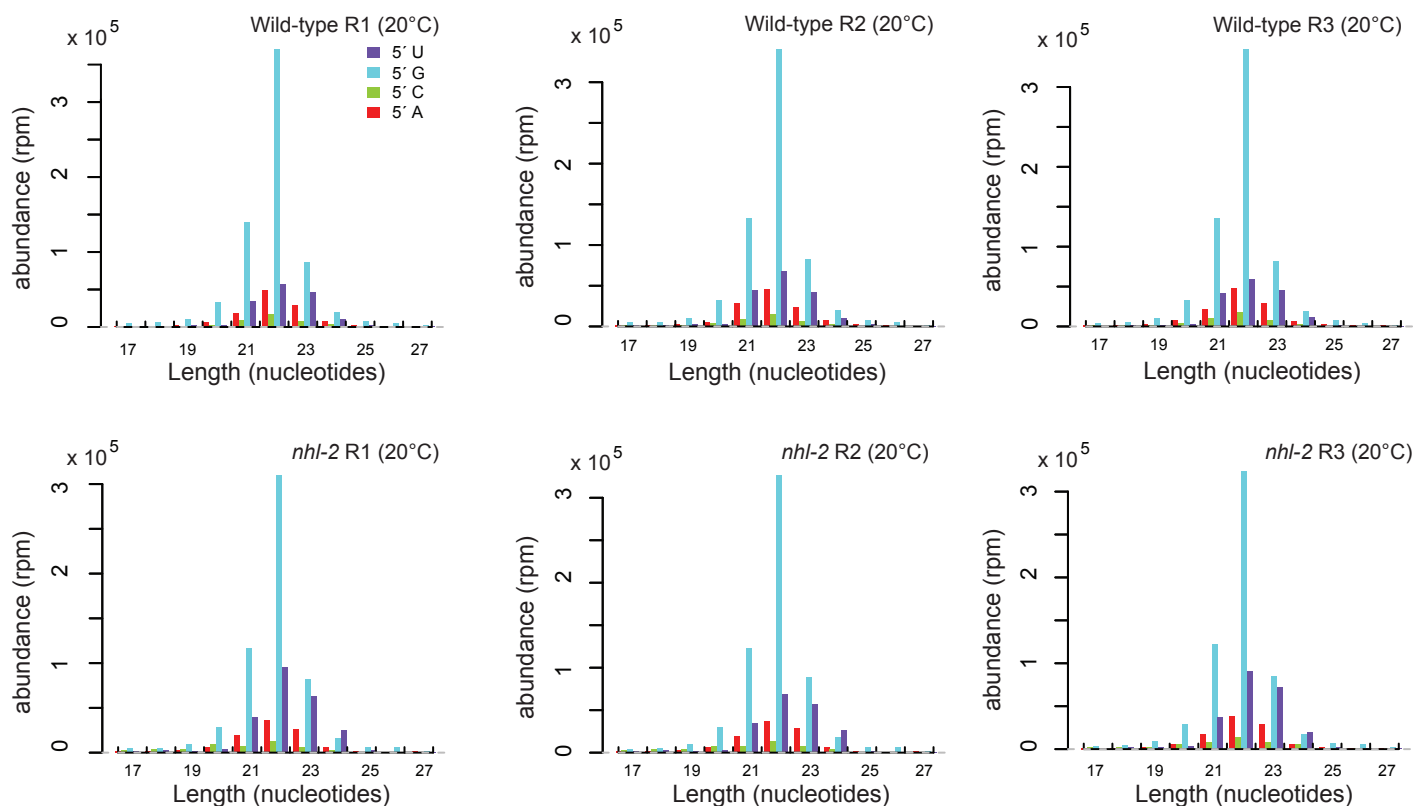
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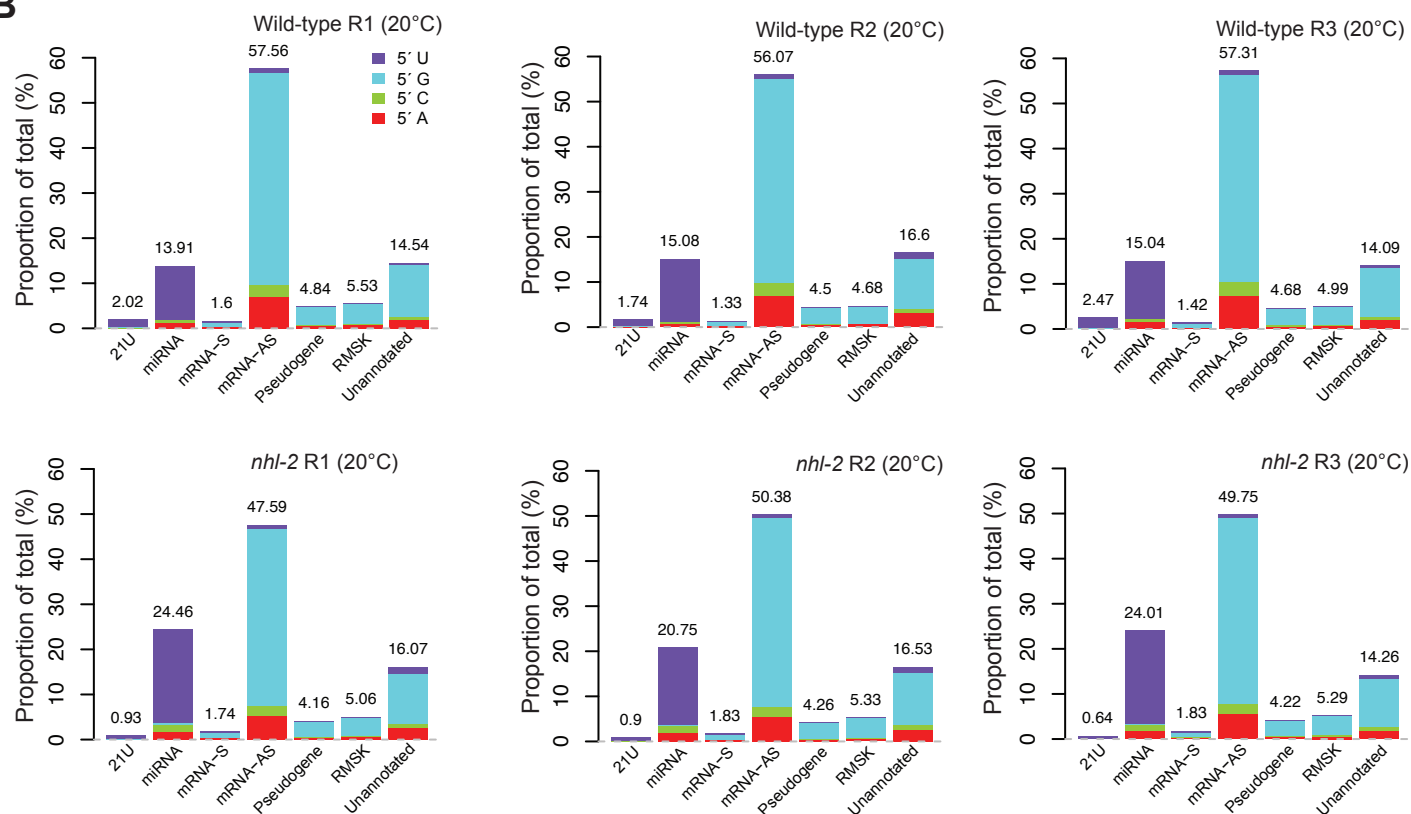


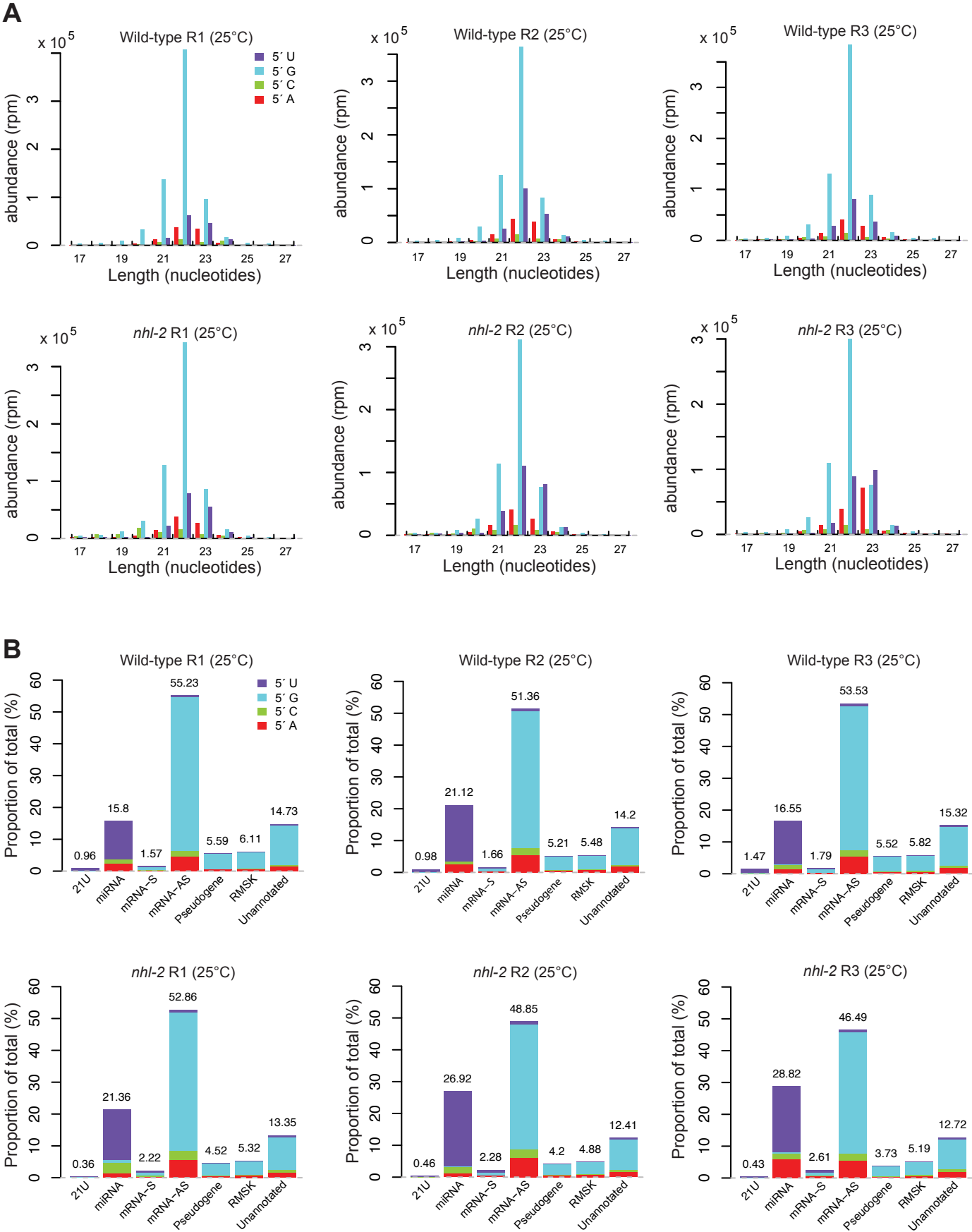
Davis Supplemental Fig. S5

A

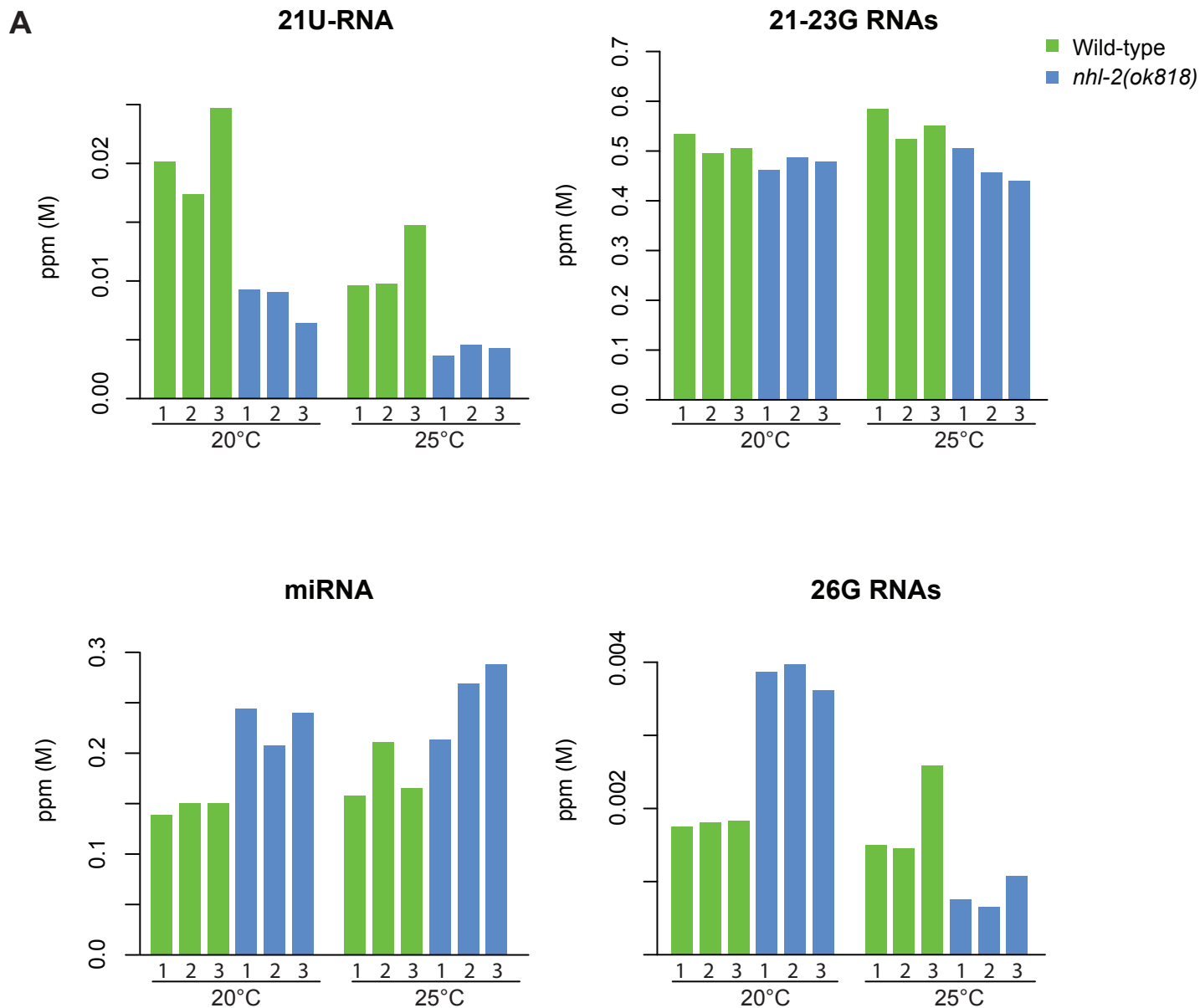


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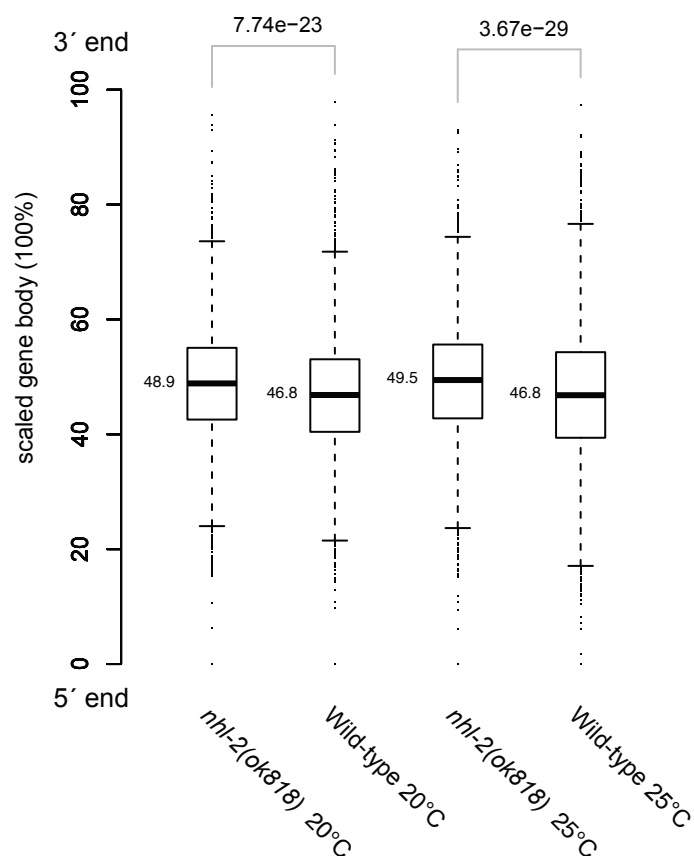


Davis Supplemental Fig. S7

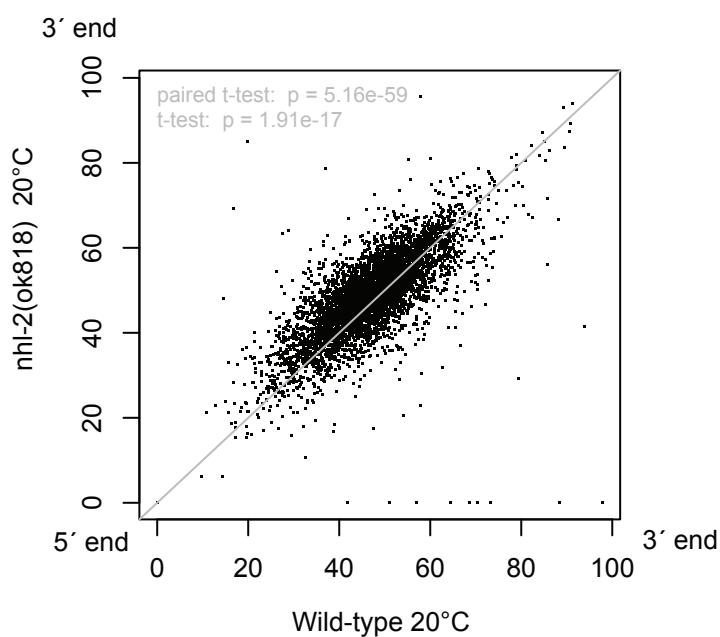


Davis Supplemental Fig. S8

A



B



C

