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TRIM-NHL protein, NHL-2, modulates cell fate choices in the *C. elegans* germ line

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Many tissues contain multipotent stem cells that are critical for maintaining tissue function. In *Caenorhabditis elegans*, germline stem cells allow gamete production to continue in adulthood. In the gonad, GLP-1/Notch signaling from the distal tip cell niche to neighboring germ cells activates a complex regulatory network to maintain a stem cell population. GLP-1/Notch signaling positively regulates production of LST-1 and SYGL-1 proteins that, in turn, interact with a set of PUF/FBF proteins to positively regulate the stem cell fate. We previously described sog (suppressor of glp-1 loss of function) and teg (tumorous enhancer of glp-1 gain of function) genes that limit the stem cell fate and/or promote the meiotic fate. Here, we show that sog-10 is allelic to nhl-2. NHL-2 is a member of the conserved TRIM-NHL protein family whose members can bind RNA and ubiquitinate protein substrates. We show that NHL-2 acts, at least in part, by inhibiting the expression of PUF-3 and PUF-11 translational repressor proteins that promote the stem cell fate. Two other negative regulators of stem cell fate, CGH-1 (conserved germline helicase) and ALG-5 (Argonaute protein), may work with NHL-2 to modulate the stem cell population. In addition, NHL-2 activity promotes the male germ cell fate in XX animals.

1. Introduction

Formation of specialized cells during animal development involves a series of tightly regulated cell fate choices. Many tissues contain stem cells, which retain the ability to self-renew or give rise to cells with a more limited fate. An example is germline stem cells (GSCs) in the nematode, *Caenorhabditis elegans* (reviewed by Hubbard and Schedl, 2019). The *C. elegans* gonad is organized as a reeled tube with mitotically cycling stem cells located at the distal end and mature gametes accumulating at the proximal end. The somatic distal tip cell (DTC) surrounds the distal end of the germ line and provides a niche, communicating with germ cells via Notch-type signaling to specify the stem cell fate and prevent those cells from entering meiosis (Fig. 1). The germline expresses a Notch-type receptor called GLP-1, so named because mutants have a germline proliferation defective phenotype (Austin and Kimble, 1987). Immediately proximal to the GSCs is a population of cells completing a final mitotic cell cycle, and further proximal is a population of non-cycling cells in meiotic S phase (Hansen et al. 2004; Crittenden et al. 2006; Maciejowski et al. 2006; Jaramillo-Lambert et al. 2007; Fox et al. 2011; Fox and Schedl, 2015; Seidel and Kimble, 2015). These three populations are collectively called the progenitor zone. As cells complete meiotic S phase and move further proximally away from the niche, they enter meiotic prophase I under control of several meiotic entry pathways. When GLP-1/Notch signaling is impaired, some or all germline stem cells will prematurely exit mitosis and enter meiosis, indicating that this signaling maintains the GSC fate. Hence, the *glp-1* loss of function phenotype is a premature switch from stem cell fate to meiotic fate. Although GLP-1/Notch signaling maintains the GSC population, it is important to note that it does not appear to regulate the frequency or length of mitotic cycling in these cells. A network of factors acts downstream of GLP-1 to regulate cell fate in the distal germ line. GLP-1/Notch signaling directly upregulates transcription of two genes, *lst-1* and *sygl-1*, whose protein products act together with four PUF (PuBFRF) family mRNA-binding proteins, FBF-1 (*fem-3* binding factor), FBF-2, PUF-3, and PUF-11, to promote the GSC fate (Kershner et al. 2014; Lee et al., 2016; Brenner and...
implicated in regulating gene expression at multiple post-transcriptional levels (Hyenne et al., 2008; McJunkin and Ambros, 2017; Davis et al. 2018). TRIM-NHL proteins function in diverse animal species where they regulate developmental fate choices (Tocchini and Ciosk, 2015; Connacher and Goldstrohm, 2021). Characteristic features of this protein family are (i) a TRIPartite Motif (TRIM), comprising a RING domain, B-Box-type zinc fingers, and a coiled-coil domain that functions in ubiquitinating target proteins, and (ii) a C-terminal NHL domain with RNA-binding capability (Hyenne et al. 2008; Tocchini and Ciosk, 2015; Connacher and Goldstrohm, 2021). We identify distinct germline-autonomous functions for NHL-2 in two fate choices: limiting the GSC fate and/or promoting differentiation (meiotic entry), at least in part through inhibition of PUF-3 and PUF-11 accumulation; and promoting the male germ cell fate in the larval XX hermaphrodite germline. We identify two other negative regulators of the GSC fate, CGH-1 (conserved germline helicase) and ALG-5 (argonaute-like gene), that may work with NHL-2 to regulate the GSC vs meiotic choice.

2. Materials and Methods

2.1. Genetics

Strains were cultured using standard methods. The C. elegans Bristol variant (N2) and mutations used are listed in Wormbase or described in the text. The following mutations were used. LG (linkage group) I: alg-5(m1163), alg-5(gk19870), alg-5(gk707311), gld-1(q485), glb-2(q497), rfy-1(pk1417). LGII: fbf-1(ok91), fbf-2(q704), glb-3(q730), nos-3(oz231). LGIII: cgh-1(ok492), dpy-17(e164), glp-1(q224ts), glp-1(ar202gf), glp-1(ok264gf), nhl-2(ok818), pal-1(e224), sog-10(q162), unc-32(e189). LG IV: rde-1(e219). We used the GFP-expressing balancers htl(4(e937) let-7(q782) q648) and mlh1[dpv-10(e128) M14]. CRISPR-Cas9 gene-edited [tl-1::3xflag and 3xflag:sgyl-1 are described in Kociossova et al. (2019), and 3xV5::pu5-3 and 3xV5::pu5-11 are described in Haupt et al. (2020)]. A strain expressing germline rde-1(–) is described below. We use the abbreviation (0) to designate the canonical null allele of a gene, and otherwise specify the allele used.

2.2. Whole genome sequencing

Genomic DNA isolation from sog-10(q162) homozygous mutants, library construction, whole genome sequencing (WGS), and bioinformatics analysis were performed as described (Rastogi et al. 2015). 3-factor mapping prior to WGS placed q162 within the pal-1 – dpy-17 interval on LGIII. Therefore, we particularly focused on WGS data from this region. Two coding changes were detected in the pal-1 – dpy-17 interval.
and three coding changes were detected in nearby flanking regions (Fig. 1A). These coding changes were as follows: open reading frame (ORF) B0285.4, nucleotide (nt) 4345012 G→T, amino acid (aa) A→S; him-18/7044A.15, nt 4712954 C→G, aa W→C; fkh-5/F26A1.2, nt 4852318 A→T, aa N→I; nhl-2/F26F4.7, nt 4896956 C→T, aa W→stop; and K10D2.1, nt 5195035 C→A, aa T→K.

2.3. RNAi

RNAi was performed by the standard feeding method adapted from Timmons et al. (2001). glp-1(q224) or glp-1(q224ts) L1 animals at 20 °C were fed single E. coli strains containing bacterial plasmids expressing dsRNA corresponding to candidate genes in the mapped LGIII interval, and then scored as adults. Germline-specific RNAi was performed with a strain that expresses RDE-1, an Argonaute protein required for RNAi, under a germline mex-5 promoter (mex-5:rade-1(+); rde-1(ne219)) (Marre et al. 2016). In addition, RNAi experiments were also performed in a runf(0) background where RNAi is disabled in many somatic tissues, including the somatic gonad (Kumsta and Hansen, 2012).

2.4. Embryo assays

Embryo counts were obtained by standard methods. Temperature-sensitive glp-1(q224), nhl-2(ok162) glp-1(q224), nhl-2(ok818) glp-1(q224), and alf-5(tm1163);glp-1(q224) strains were maintained at 15 °C. To assay for embryo production, L1 larvae were picked to individual 20 °C plates and maintained at 20 °C throughout the experiment. If individuals developed as gravid adults, they were moved to fresh plates daily, and embryos present on the plates were counted. Plates were checked 1 and 2 days later to determine if any embryos hatched as larvae.

2.5. Immunocytochemistry

Antibodies were kindly provided by the following colleagues: anti-NHL-2 from the Jantch/Loidl lab; and anti-MSP from the Greenstein lab. Anti-V5-Tag antibody was purchased from Bio-Rad, anti-WAPL-1 from Timmons et al. (2001). gfp; nhl-2(ok818) glp-1(q224)/hT2 gfp animals were obtained for the embryo assay.

3. Results

3.1. sog-10(q162) is allelic to nhl-2

The sog-10(q162) mutation partially suppresses the premature meiotic entry phenotype of glp-1 partial loss-of-function (lf) mutations (Maine and Kimble, 1993). It is unique among described sog mutations in that it does not suppress the glp-1(lf) embryonic lethal phenotype. We set out to identify the causative mutation in q162. Traditional three-point mapping placed q162 in a region on LGIII between pal-1 and dpy-17. Whole genome sequencing revealed five coding region mutations within or near the pal-1 to dpy-17 region (Fig. 2A) (see Methods). We assayed for suppression of the premature meiotic entry phenotype by knocking down these five genes individually by RNAi in glp-1(q224) animals cultured at 20 °C. At 20 °C, the wildtype hermaphrodite germline produces ~300 sperm during larval development before switching to oogenesis at the adult molt and subsequently producing embryos. The glp-1(q224) mutation is temperature-sensitive (ts); when mutant hermaphrodites are raised at 20 °C, their germ cell precursors all enter meiosis prematurely and undergo spermatogenesis, and hence these animals do not produce oocytes or embryos. We asked whether RNAi-mediated knockdown of any of the five candidate sog-10 genes could suppress the glp-1(q224) meiotic entry defect sufficiently to allow production of embryos. Among the five genes tested, only knockdown of nhl-2 resulted in suppression, where glp-1(q224) hermaphrodites produced embryos at 20 °C. These nhl-2(RNAi) glp-1(q224) embryos did not hatch, indicating the glp-1(lf) embryonic lethality was not suppressed by nhl-2(RNAi).

The nhl-2 coding sequence isolated from the q162 strain contains a C to T transition that changes a tryptophan residue to a premature stop codon in exon 5, approximately halfway through the protein-coding region (Fig. 2B). This change is likely to cause a loss of gene function since no meiotic entry defect is observed in glp-1(q224) larvae. We conclude that the premature meiotic entry defect is suppressed by nhl-2(RNAi). We also observed an incompletely penetrant Fog phenotype in nhl-2(ok818) XX animals raised at 15 °C (Fig. 2C-E). We conclude that sog-10(q162) is a strong loss-of-function allele of nhl-2. We will refer to q162 as an allele of nhl-2 for the remainder of the paper.
allowing production of embryos at 20°C of individual hermaphrodites assayed. Results for upshifted to 20°C/C14/C14 assays reported here, most strains were maintained at 15°C. Germ cells prematurely enter meiosis during early larval development. For the 5(tm1163);nhl-2(ok818) glp-1(q224) animals, which were derived from embryonic germ lineage, NHL-2 has been detected both as diffuse within cells, including the adult hermaphrodite germline and all, cells throughout development (Hammell et al. 2009; Hyenne et al., 2008). Within cells, NHL-2 is expressed throughout germ cell development and 2008). Within cells, including the adult hermaphrodite germline and otherwise retain a standard germline with meiotic nuclei gonads show relatively mild extension of the progenitor zone compared to wildtype and otherwise retain a standard germline with meiotic nuclei (Pepper et al. 2003). At glp-1(ar202gf) mutant gonads show a strong tumorous germline phenotype of nhl-2. Since mutants, we predicted that nhl-2 function could enhance the tumorous phenotype of nhl-2. (B) nhl-2 gene model. Blue boxes represent exons, intersecting lines represent introns. Relative positions of conserved domains are indicated below: a RING domain (green), a ZnF/B-Box (magenta), a C-terminal B-Box (yellow), and several NHL-repeats (orange, with a single NHL repeat in red to show scale). q162 is a nonsense mutation at amino acid residue 484 (cryptophan). The ok818 deleted region is indicated with a bracket. (C) Representative image of wildtype hermaphrodite gonad at young adult stage, ~4 h post-L4 molt, at 15°C. Spermatids are present in the proximal gonad arm and contain major sperm proteins (MSP) as detected by immunolabeling. (D) Representative image of a nhl-2(ok818) hermaphrodite gonad ~4 h post-L4 molt at 15°C displaying a feminized germline (Fog) phenotype. NHL-2 was low or absent in germ cells that were visibly undergoing spermatogenesis (Fig. 3C). Consistent with published reports, we observed diffuse NHL-2 signal and small cytoplasmic puncta, including some that overlapped with perinuclear P granules (Fig. 3G).

3.3. NHl-2 inhibits germ stem cell fate and/or promotes meiotic entry

Since nhl-2 mutations suppressed the premature meiotic entry phenotype of gfp-1(q224) mutants, we predicted that nhl-2 activity may normally inhibit the GSC fate and/or promote meiotic entry. To explore further, we tested if loss of nhl-2 function could enhance the tumorous phenotype of a weak gfp-1 gain-of-function (gf) allele, ar202. At 25°C, gfp-1(ar202gf) mutant gonads show a strong tumorous germline phenotype, and most germ cells fail to enter meiosis (Pepper et al. 2003). At semi-permissive temperatures of 15°C and 20°C, gfp-1(ar202gf) mutant gonads show relatively mild extension of the progenitor zone compared to wildtype and otherwise retain a standard germline with meiotic nuclei and gametes (Hansen et al. 2004) (Fig. 4A). For this assay, we identified the progenitor zone by immunolabeling REC-8, a sister chromatid cohesion protein, under fixation conditions allowing its detection only in the cytoplasm and in discrete cytoplasmic puncta (Hyenne et al. 2008; Davis et al. 2018) (Fig. 3). P granules are ribonucleoprotein (RNP) condensates and contain diverse RNA regulatory proteins, mRNAs, and small RNAs (sRNAs) and thought to be sites of extensive RNA regulation (Wang and Seydoux, 2013; Seydoux, 2018). We evaluated the distribution of NHL-2 in the developing germ line using immunolabeling. NHL-2 appeared mildly enriched in proliferating germ cells at all stages of larval development, with its level dropping modestly as germ cells appeared mildly enriched in proliferating germ cells at all stages of larval development, with its level dropping modestly as germ cells entered meiotic prophase (Fig. 3A–F). NHL-2 was low or absent in germ cells that were visibly undergoing spermatogenesis (Fig. 3C). Consistent with published reports, we observed diffuse NHL-2 signal and small cytoplasmic puncta, including some that overlapped with perinuclear P granules (Fig. 3G).

Table 1: Loss of nhl-2 function suppresses the gfp-1(q224ts) germline proliferation defect allowing production of embryos at 20°C.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>No. embryos produced ± SEM (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gfp-1(q224)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>nhl-2(q162);glp-1(q224)</td>
<td>10</td>
<td>124 ± 18 (46–182)</td>
</tr>
<tr>
<td>nhl-2(ok818);glp-1(q224)</td>
<td>9</td>
<td>112 ± 13 (56–180)</td>
</tr>
<tr>
<td>alg-5(m1163);glp-1(q224)</td>
<td>11</td>
<td>8 ± 1.5 (1–18)</td>
</tr>
<tr>
<td>alg-5(m1163);nhl-2(ok818);glp-1(q224)</td>
<td>19</td>
<td>117 ± 9 (0–166)</td>
</tr>
</tbody>
</table>

gfp-1(q224ts) hermaphrodites grown at 15°C produce offspring. When raised at 20°C, they produce only a few sperm and no oocytes or embryos because all germ cells prematurely enter meiosis during early larval development. For the assays reported here, most strains were maintained at 15°C, and L1 larvae were upshifted to 20°C to assay for embryo production. The exception was alg-5(m1163);nhl-2(ok818);glp-1(q224) animals, which were derived from alg-5(m1163);nhl-2(ok818);glp-1(q224)/HT2 gfp hermaphrodites grown at 20°C. Results for nhl-2(q162) are consistent with Maine and Kimble (1993). N, number of individual hermaphrodites assayed.

Embryos do not survive, indicating that the loss of NHL-2 function does not suppress gfp-1(ts) maternal-effect embryonic lethality.

3.2. NHL-2 is expressed throughout germ cell development

NHL-2 is a cytoplasmic protein ubiquitously expressed in most, if not all, cells throughout development (Hammell et al. 2009; Hyenne et al., 2008). Within cells, including the adult hermaphrodite germline and embryonic germ lineage, NHL-2 has been detected both as diffuse within...
the nucleoplasm, and we identified meiotic nuclei by immunolabeling HIM-3, an axial component of the synaptonemal complex that associates with chromosomes in first meiotic prophase (Hansen et al. 2004).

*glp-1(ar202gf)* mutants are sensitive to loss of gene products that inhibit the stem cell fate and/or promote meiotic entry (e.g., MacDonald et al. 2008; Chen and Greenwald, 2015; Safdar et al. 2016). We examined gonads from *nhl-2(ok818) glp-1(ar202gf)* double mutants and found that only a few germ cells had entered meiosis in 1-day old adults raised at 15°C, and essentially no germ cells had entered meiosis in 1-day old adults raised at 20°C (Fig. 4B). At 20°C, 15/16 gonads scored contained no HIM-3 positive nuclei; all germline nuclei were REC-8 positive. 1/16 gonads had a very few HIM-3 positive nuclei; all other nuclei were REC-8 positive. This strong enhancement of the *glp-1(ar202gf)* tumorous phenotype is consistent with NHL-2 acting to inhibit the GSC fate and/or promote meiotic entry. To identify a more specific role for NHL-2, we examined the effect of *nhl-2(ok818)* on different components of the GSC gene regulatory network (Fig. 1), as described below.

### 3.4. LST-1 and SYGL-1 accumulation is unchanged in the *nhl-2(ok818)* background

GLP-1/Notch signaling induces *lst-1* and *sygl-1* transcription in the distal most ~5 cell diameters adjacent to the DTC, and LST-1 and SYGL-1 proteins together are required to prevent meiotic entry (Kershner et al. 2014; Lee et al. 2016; Haupt et al. 2020; Chen et al. 2020). Based on NHL-2 inhibiting the GSC fate, we asked whether NHL-2 might regulate expression of LST-1 and SYGL-1 accumulation within the distal end of the germ line. We evaluated *LST-1::3xFLAG* and *3xFLAG::SYGL-1* accumulation levels in *nhl-2(+)* controls and *nhl-2(ok818)* germlines. For each protein, the overall accumulation pattern was remarkably similar between *nhl-2(+)* and *nhl-2(ok818)*, except for a slight, although statistically significant, increase at two points in the peak levels for both LST-1 and SYGL-1 (Fig. 5). Importantly, there was no significant increase at proximal positions, indicating that the length of the stem cell pool in *nhl-2(ok818)* is the same as wildtype. Therefore, NHL-2 is unlikely to regulate LST-1 and/or SYGL-1 accumulation.

### 3.5. *nhl-2(ok818)* suppresses the premature meiotic entry phenotype of *fbf-1(0) fbf-2(0)*

We next examined the Pumilio-related genes, *fbf-1*, *fbf-2*, *puf-3* and *puf-11*, which function in conjunction with GLP-1 targets, *sygl-1* and *lst-1*, to repress the meiotic entry pathway genes (Fig. 1). At low culture temperatures (15°C–20°C), *fbf-1(0) fbf-2(0)* double mutants have a late-onset Glp-1-like phenotype where all GSCs prematurely enter meiosis during late larval development and form sperm (Crittenden et al. 2002).
very few meiotic nuclei. Scale bars, 50 μm. (B) Representative gfp-1(ar202gf) gonad containing ectopic REC-8 positive (progenitor) nuclei intermixed with patches of HIM-3 positive (meiotic) nuclei. (C) Representative gfp-1(ar202gf) gonad containing ectopic REC-8 positive (progenitor) nuclei intermixed with patches of HIM-3 positive (meiotic) nuclei. (D) Representative gfp-1(ar202gf) gonad containing ectopic REC-8 positive (progenitor) nuclei intermixed with patches of HIM-3 positive (meiotic) nuclei. 

(A) Representative gfp-1(ar202gf) gonad at 20 °C. Progenitor zone nuclei have prominent REC-8 label, and meiotic prophase nuclei have prominent HIM-3 signal. (B) Representative ngl-2(ok818) gfp-1(ar202gf) gonad contains a germ-line tumor with essentially no meiotic nuclei. (C) Representative ngl-2(ok818), gfp-1(ar202gf) gonad containing ectopic REC-8 positive (progenitor) nuclei intermixed with patches of HIM-3 positive (meiotic) nuclei. (D) Representative ngl-2(ok818), gfp-1(ar202gf) gonad containing ectopic REC-8 positive (progenitor) nuclei intermixed with patches of HIM-3 positive (meiotic) nuclei.

Fig. 4. Loss of NHL-2 function enhances the weak glp-1(gf) tumorous phenotype. Images show dissected gonads from 1-day old adult hermaphrodites raised at 20 °C that are stained with DAPI to visualize DNA (top) and immunolabeled with anti-REC-8 (green, bottom) and anti-HIM-3 (red, bottom) antibodies (+, distal end of the gonad arm; gonads are outlined with a dotted line. (A) Representative gfp-1(ar202gf) gonad at 20 °C. Progenitor zone nuclei have prominent REC-8 label, and meiotic prophase nuclei have prominent HIM-3 signal. (B) Representative ngl-2(ok818), gfp-1(ar202gf) gonad contains a germ-line tumor with essentially no meiotic nuclei. (C) Representative ngl-2(ok818), gfp-1(ar202gf) gonad containing ectopic REC-8 positive (progenitor) nuclei intermixed with patches of HIM-3 positive (meiotic) nuclei. (D) Representative ngl-2(ok818), gfp-1(ar202gf) gonad containing ectopic REC-8 positive (progenitor) nuclei intermixed with patches of HIM-3 positive (meiotic) nuclei. 

(Fig. 6A). Quadruple mutant animals, that also carry puf-3(0) and puf-11(0) mutations, have a stronger Glp-1-like null phenotype where all GSCs enter meiosis in early larval development, supporting their redundant function in promoting the stem cell fate (Haupt et al. 2020). We tested whether ngl-2(ok818) could suppress the late-onset fbf-1(0) fbf-2(0) Glp-1-like phenotype by evaluating the germlines of fbf-1(0) fbf-2(0); ngl-2(ok818) triple mutant raised at low temperature. 100% of fbf-1(0) fbf-2(0); ngl-2(ok818) adult hermaphrodite germlines contained not only mature sperm, but also primary spermatocytes (indicating active spermatogenesis) and distal cells in meiotic prophase and/or mitotically cycling like cells (Fig. 6A). These results indicate that ngl-2(ok818) partially suppresses the fbf-1(0) fbf-2(0) premature meiotic entry defect in late larvae/early adults.

To further analyze the extent to which ngl-2(ok818) suppresses the fbf-1(0) fbf-2(0) genotype, we immunolabeled HIM-3. All fbf-1(0) fbf-2(0); ngl-2(ok818) germlines contained nuclei just distal to the primary spermatocytes where chromosome-associated HIM-3 was evident (n = 14), and most germlines contained progenitor zone-like nuclei without chromosome-associated HIM-3 at the distal end of the gonad arm (n = 13/14) (Fig. 6B). These results confirm that meiotic prophase nuclei are present in fbf-1(0) fbf-2(0); ngl-2(ok818) germlines at an age when they are no longer present in fbf-1(0) fbf-2(0) controls and, in addition, fbf-1(0) fbf-2(0); ngl-2(ok818) adults likely retain some GSCs. The suppression results are consistent with ngl-2 opposing fbf-1 and fbf-2 activity, either directly or indirectly.

3.6. NHL-2 is a negative regulator of PUF-3 and PUF-11 accumulation in the distal germline

To examine whether PUF-3 and/or PUF-11 accumulation is altered in the ngl-2(ok818) mutant, we compared PUF expression in ngl-2(ok818) and ngl-2(+ ) germ lines. For these experiments, we assayed 3xV5::PUF-3 and 3xV5::PUF-11 produced from epitope-tagged endogenous alleles (Haupt et al. 2020) (see Methods). In wildtype adult hermaphrodites, PUF-3 and PUF-11 are present at relatively low levels in the progenitor zone and much higher levels in the proximal late meiotic prophase germ line (Haupt et al. 2020). We focused on the progenitor zone and quantified PUF expression across the distal 35 germ cell diameters (Fig. 7, Figs. S1 and S2). We observed a statistically significant increase in both PUF-3 and PUF-11 expression in the distal ngl-2(ok818) germ line compared to controls (Fig. 7A, Fig. S1). PUF-3 was significantly more abundant in ngl-2(ok818) compared to wildtype across the entire 35 distal germ cells, with the largest increase (~60%) at 5 cell diameters from the distal tip. In contrast, PUF-11 was significantly more abundant in ngl-2(ok818) compared to wildtype across the distal 13 germ cells and then dropped below wildtype level (Fig. 7A, Figs. S1 and S2). PUF-11 peaked more distally in ngl-2(ok818) (10–13 cell diameters) than in wildtype (19–21 cell diameters). At 5 cell diameters, PUF-11 was ~30% more abundant in ngl-2(ok818) than in wildtype. These results indicate that NHL-2 is a negative regulator of PUF-3 and PUF-11 accumulation in the progenitor zone. The increase in both PUF-3 and PUF-11 expression in ngl-2(ok818) provides an explanation, at least in part, for the suppression observed in the fbf-1(0) fbf-2(0) paralog double mutant, as well as the suppression of glp-1(g224) and the enhancement gfp-1(ar202gf).

3.7. NHL-2 does not appear to act in or downstream of the meiotic entry pathways

LST-1 and SYGL-1, together with FBF-1, FBF-2, PUF-3, and PUF-11, ensure the GSC fate by repressing the activity of three meiotic entry pathways mediated by GLD-1 (mutants have defective germ line development), GLD-2, and SCFPROM-1. (ubiquitin ligase complex) (Fig. 1). These pathways repress translation of GSC factors, promote translation of meiotic factors, and promote turnover of progenitor zone proteins, respectively (Hubbard and Schell, 2019). In single GLD-1, GLD-2, and SCFPROM-1 pathway mutants, meiotic entry occurs relatively normally. However, in animals carrying mutations in any two pathways, e.g., glp-1(q224) gld-1(q485) double mutants, meiotic entry is impaired, and a synthetic germline tumor develops (Kadyk and Kimble, 1998; Moham- mad et al., 2018). One hypothesis for ngl-2(ok818) suppression of glp-1(q224) and fbf-1(0) fbf-2(0), and the enhancement gfp-1(ar202gf), is that NHL-2 functions in or downstream of one or more of these meiotic entry pathways - perhaps in addition to its role in regulating PUF
expression. We tested this idea by making double mutants carrying nhl-2(ok818) and a GLD-1 pathway mutant [gld-1(q485), n = 30] or GLD-2 pathway mutant [gld-2(q497), n = 27; gld-3(q730), n = 22] and did not observe a synthetic tumor in any case. These results suggest that NHL-2 does not function in a meiotic entry pathway. To look more closely at a potential role for NHL-2 in meiotic entry, we used a sensitized genetic background where meiotic entry is weakly impaired. The synthetic germline tumor in gld-2(q497); nos-3(oz231) mutants is mild compared with gld-2(q497) gld-1(q485) mutants (Hansen et al., 2004; Mohammad et al., 2018). Adult gld-2(q497);nos-3(oz231) germlines have substantial numbers of meiotic nuclei whereas gld-2(q497) gld-1(q485) germlines have very few meiotic nuclei. We compared the synthetic tumor in gld-2(q497);nos-3(oz231) double mutants and gld-2(q497);nos-3(oz231);nhl-2(ok818) triple mutants (Fig. S3). We observed a similar distribution of REC-8 positive and HIM-3 positive nuclei in gld-2(q497);nos-3(oz231) [n = 16] and gld-2(q497);nos-3(oz231);nhl-2(ok818) [n = 15] gonad arms. Hence, nhl-2(ok818) did not obviously enhance the synthetic tumor. We interpret these results to indicate that NHL-2 likely does not act in or downstream of the GLD-1, GLD-2, or SCFPROM/1 meiotic entry pathway(s). Instead, NHL-2 may modulate progenitor zone size by acting solely upstream of these pathways.

3.8. NHL-2 promotes the normal size adult progenitor zone

In the C. elegans gonad, GLP-1/Notch signaling maintains germline stem cells, i.e., ensuring they do not differentiate, while nutritional and other inputs ensure robust mitotic proliferation of these cells (Singh and Hansen, 2017; Hubbard et al., 2013). During our studies, we noticed that nhl-2 mutant adults had a smaller progenitor zone compared to nhl-2(+) controls based on marker protein analysis. In WAPL-1 staining experiments, the adult nhl-2(ok818) progenitor zone was shorter than in wild-type controls (17–18 vs 20 gcd) (Fig. 5; Fig. S2). Furthermore, the total number of cells in the nhl-2(ok818) progenitor zone was about 2/3 the number in wild type (Fig. S4), demonstrating a significant reduction in progenitor zone size. This nhl-2 mutant phenotype seems counter to suppression of premature meiotic entry in glp-1(q224) partial loss of function and enhancement of the tumorous phenotype in glp-1(ar202gf). Similarly, puf-8(0) is also an enhancer of glp-1(ar202gf) as well as promotes the normal size of the progenitor zone (see Introduction). The size of the progenitor zone is a complex interaction of multiple processes, including the size of the stem cell pool, the rate of mitotic cell cycling, the rate of meiotic entry, and progenitor zone expansion during larval development, which is regulated by worm physiology (Hubbard and Schedl, 2019). Additional studies will be necessary to determine the basis of the smaller progenitor zone in nhl-2 mutants, although the LST-1 and SYGL-1 immunolabeling data (Fig. 5) suggest that it is not an effect of the stem cell pool.

3.9. nhl-2 RNAi phenotypes suggest germline specific NHL-2 functions

Because NHL-2 is widely expressed in most tissues throughout development, we wanted to determine if it acts specifically in germ cells to regulate germ cell processes. To address this question, we used a transgenic strain to RNAi knockdown nhl-2 expression in germ cells and not in the somatic gonad (see Materials and Methods for details).
mediated repression of certain mRNAs in somatic tissues (Hammell et al. 2009; Alessi et al. 2015). CGH-1 is member of the conserved DDX6 family whose members are key components of RNA processing (P) bodies and participate in translational regulation and mRNA turnover in various organisms (reviewed by Rajayaguru and Parker, 2009). NHL-2 associates physically with CGH-1 as well as the miRNA Argonaute proteins, ALG-1 and ALG-2, in co-IP experiments and co-localizes with CGH-1 in protein-RNA condensates in germline and somatic tissues (Hammell et al. 2009; Davis et al. 2018). These findings prompted us to ask if NHL-2 might function together with CGH-1 or components of the core miRNA machinery in modulating the GSC fate vs meiotic entry decision. To examine this question, we first generated a cgh-1(ok492) glp-1(ar202gf) double mutant strain and evaluated the miRNA entry phenotype in adults at permissive temperature (20 °C) by immunostaining for REC-8 and HIM-3. As described above, glp-1(ar202gf) control germlines at 20 °C typically contain not only proliferative nuclei, but contain meiotic prophase nuclei as well. In contrast, for 5/48 cgh-1(ok492) glp-1(ar202gf) gonad arms, all germ cell nuclei were REC-8 positive and HIM-3 negative, indicating none had entered meiosis. Another 40/48 gonad arms included ectopic REC-8 positive nuclei intermixed with HIM-3 positive nuclei (Fig. 4C). Based on these data, the glp-1(ar202gf) weak tumorous phenotype was significantly enhanced in the cgh-1(ok492) glp-1(ar202gf) double mutant at 20 °C, consistent with CGH-1 acting to inhibit the stem cell fate and/or promote meiotic entry. Unfortunately, genetic experiments to evaluate if CGH-1 and NHL-2 act in a common pathway were not informative because (i) cgh-1(ok492) nhl-2(ok818) double mutants die at the L4 to adult molt [therefore we cannot test cgh-1 nhl-2 for further suppression of glp-1(q224)], and (ii) the nhl-2(ok818) glp-1(ar202gf) tumorous phenotype was completely penetrant [therefore we cannot test for further enhancement by cgh-1(RNAi)]. Although no germline miRNAs have yet been identified as essential for the stem cell fate vs meiotic development decision (McEwen et al. 2016; Brown et al. 2017; Minogue et al. 2018; Dallaire et al. 2018; Theil et al. 2019), we decided to explore this possibility directly by testing if the glp-1(ar202gf) weak tumorous phenotype was enhanced by loss or regulated by NHL-2.

Fig. 6. Loss of NHL-2 suppresses the fbfi-1 fbfi-2 premature meiotic entry defect. (A) Intact adult hermaphrodites were fixed at 24 h post-L4 stage and DAPI-stained to visualize DNA. One representative gonad arm is shown, * distal end of the gonad arm; p, proximal gonad. In the image labels, fbfi-1(0) indicates fbfi-1(0) fbfi-2(0), and nhl-2(0) indicates nhl-2(ok818). Left, fbfi-1(0) fbfi-2(0) germlines contain only mature sperm (arrows), whereas right, fbfi-1(0) fbfi-2(0); nhl-2(ok818) germlines contain mature sperm (arrows), primary spermatocytes (arrowheads), and additional, progenitor zone-like distal germ cells (bar). We note that fbfi-1(0) fbfi-2(0); nhl-2(ok818) triple mutants were Mof (see text). fbfi-1(0) fbfi-2(0) [n = 36] and fbfi-1(0) fbfi-2(0); nhl-2(ok818) [n = 58]. (B) Representative images of dissected adult hermaphrodite gonads stained with DAPI (upper) and labeled with anti-HIM-3 (lower, gold) at 24 h post-L4 stage. * distal end of the gonad arm. Images are maximum projections of Z-stacks processed by deconvolution (see Methods). Arrows indicate the distal most nuclei containing labeled HIM-3, and the corresponding DAPI stained nuclei. nhl-2(0) and nhl-2(ok818) germlines contained a distal progenitor zone where HIM-3 was not detected on chromosomes, as expected; nuclei located proximal to this region contain chromosome-associated HIM-3, consistent with first meiotic prophase. In fbfi-1(0/20); nhl-2(ok818) germlines, HIM-3 was detected on chromosomes in nuclei distal to the primary spermatocytes, indicating they are in first meiotic prophase. Most germlines also contained progenitor zone-like nuclei at the distal end of the gonad arm where HIM-3 chromosomal staining was not observed. We note that occasionally nhl-2(ok818) controls and fbfi-1(0) fbfi-2(0); nhl-2(ok818) triple mutants had one or a few nuclei with chromosome-associated HIM-3 within the progenitor zone and/or without chromosome-associated HIM-3 within the pachytenic zone. Scale bars, 10 μm.

3.10. CGH-1 RNA helicase and ALG-5 Argonaute may participate with NHL-2 to inhibit the stem cell fate and/or promote meiotic entry

Genetic and biochemical data suggest that NHL-2 interacts with core components of the miRNA-induced silencing complex (miRISC) and with the conserved germline helicase, CGH-1, to facilitate microRNA-mediated repression of certain mRNAs in somatic tissues (Hammell et al. 2009; Alessi et al. 2015). CGH-1 is member of the conserved DDX6 family whose members are key components of RNA processing (P) bodies and participate in translational regulation and mRNA turnover in various organisms (reviewed by Rajayaguru and Parker, 2009). NHL-2 associates physically with CGH-1 as well as the miRNA Argonaute proteins, ALG-1 and ALG-2, in co-IP experiments and co-localizes with CGH-1 in protein-RNA condensates in germline and somatic tissues (Hammell et al. 2009; Davis et al. 2018). These findings prompted us to ask if NHL-2 might function together with CGH-1 or components of the core miRNA machinery in modulating the GSC fate vs meiotic entry decision. To examine this question, we first generated a cgh-1(ok492) glp-1(ar202gf) double mutant strain and evaluated the miRNA entry phenotype in adults at permissive temperature (20 °C) by immunostaining for REC-8 and HIM-3. As described above, glp-1(ar202gf) control germlines at 20 °C typically contain not only proliferative nuclei, but contain meiotic prophase nuclei as well. In contrast, for 5/48 cgh-1(ok492) glp-1(ar202gf) gonad arms, all germ cell nuclei were REC-8 positive and HIM-3 negative, indicating none had entered meiosis. Another 40/48 gonad arms included ectopic REC-8 positive nuclei intermixed with HIM-3 positive nuclei (Fig. 4C). Based on these data, the glp-1(ar202gf) weak tumorous phenotype was significantly enhanced in the cgh-1(ok492) glp-1(ar202gf) double mutant at 20 °C, consistent with CGH-1 acting to inhibit the stem cell fate and/or promote meiotic entry. Unfortunately, genetic experiments to evaluate if CGH-1 and NHL-2 act in a common pathway were not informative because (i) cgh-1(ok492) nhl-2(ok818) double mutants die at the L4 to adult molt [therefore we cannot test cgh-1 nhl-2 for further suppression of glp-1(q224)], and (ii) the nhl-2(ok818) glp-1(ar202gf) tumorous phenotype was completely penetrant [therefore we cannot test for further enhancement by cgh-1(RNAi)]. Although no germline miRNAs have yet been identified as essential for the stem cell fate vs meiotic development decision (McEwen et al. 2016; Brown et al. 2017; Minogue et al. 2018; Dallaire et al. 2018; Theil et al. 2019), we decided to explore this possibility directly by testing if the glp-1(ar202gf) weak tumorous phenotype was enhanced by loss or
knockdown of microRNA biogenesis factors or effector components of the pathway. We did not observe enhancement of glp-1(ar202gf) meiotic entry defects when knocking down or using mutant alleles of alg-1, alg-2, dcr-1 (encoding Dicer endonuclease), dhh-1 (encoding miRNA biogenesis factor, Drosota), or pash-1 (encoding miRNA biogenesis factor, Pasha). Therefore, the core microRNA pathway may not act broadly to inhibit proliferative fate and/or promote meiotic entry.

In contrast to core miRNA pathway components, we observed strong enhancement of glp-1(ar202gf) when we knocked down alg-5. ALG-5 is the C. elegans Argonaute protein most closely related to ALG-1 and ALG-2, and it is expressed specifically in the germ line where it interacts with a subset of miRNAs and associates with P granules (Brown et al., 2017). We confirmed the alg-5(RNAi) result using alg-5(tm1163), an in-frame deletion that removes much of the PAZ domain responsible for binding miRNA, and two nonsense mutations from the Million Mutant collection that are predicted to be null, alg-5(gk119870) and alg-5(gk870731). All three alleles strongly enhanced the glp-1(ar202gf) phenotype. alg-5(tm1163);nhl-2(ok818) [n = 10] gonads contained almost entirely REC-8 positive germ cell nuclei with only a very few HIM-3 positive germ cell nuclei, as shown (Fig. 4D). Consistent with glp-1(ar202gf) enhancement, alg-5(tm1163) suppressed the glp-1(q224) loss-of-function phenotype in the germ line at 20 °C (Table 1). We conclude that ALG-5 promotes meiotic entry and/or inhibits the stem cell fate.

We further examined the relationship between NHL-2 and ALG-5 in promoting the stem cell fate in the alg-5(tm1163);nhl-2(ok818) glp-1(q224) triple mutant at 20 °C. Suppression of the glp-1(q224) germline phenotype in this triple mutant and the nhl-2(ok818) glp-1(q224) double mutant were similar (Table 1). This result is consistent with NHL-2 and ALG-5 acting in a common genetic pathway to promote meiotic entry and/or inhibit the stem cell fate.

4. Discussion

Here we identified sog-10(q162), a mutation previously described as suppressing a germline GLP-1/Notch signaling defect (Maine and Kimble, 1993), as a strong loss-of-function allele of nhl-2. NHL-2 is a member of the broadly conserved TRIM-NHL family of proteins that includes other known cell fate regulators, including Drosoophilia Brat (Brain tumor) and MEI-P26 (meiotic P26), mammalian TRIM3 and TRIM32, and five C. elegans proteins (Tocchini and Ciosk, 2015; Connacher and Goldstrohm, 2021). We identify several germline biological processes where NHL-2 functions, likely in an autonomous manner. NHL-2 is a negative regulator of the stem cell fate/positive regulator of meiotic entry, promotes the normal size of the progenitor zone, and has a minor role in promoting the male fate at lower culture temperatures.

Several lines of evidence support a role for NHL-2 in negatively regulating the stem cell fate. The loss of nhl-2 function suppresses the premature meiotic entry phenotype of glp-1(q224) and enhances the weak tumorous phenotype of glp-1(ar202gf). Downstream of GLP-1 signaling, four redundant Pumilio family RNA-binding proteins, FBF-1, FBF-2, PUF-3, and PUF-11, promote the stem cell fate (Haupt et al., 2020). We found that both PUF-3 and PUF-11 expression was increased in nhl-2(ok818) mutants, consistent with NHL-2 acting as a negative regulator of the stem cell fate. We also found that nhl-2(ok818) suppressed the premature meiotic entry phenotype of the fbf-1(0) fbf-2(0) double mutant. We speculate that the fbf-1(0) fbf-2(0) suppression is a result of increased PUF-3 and PUF-11 accumulation in the absence of nhl-2. Furthermore, we propose that suppression of glp-1(q224) and enhancement of glp-1(ar202gf) in nhl-2(0) are due to increased accumulation of the downstream PUF-3 and PUF-11 proteins (Fig. 9). NHL-2 acting at the level of PUF-3 and PUF-11 also explains why the embryonic lethal phenotype of glp-1(q224) was not suppressed, as the Pumilio proteins are not known to function downstream of GLP-1 signaling in the embryo. Based on the mutant phenotype, NHL-2 would appear to have a modulatory role as loss of the nhl-2 gene on its own has relatively mild germline phenotypes. However, it remains possible that there is redundancy with other TRIM-NHL family members (see below).

Increased PUF-3 and PUF-11 accumulation could potentially result in feminization of the germline, providing an explanation for the nhl-2(0) incompletely penetrant Fog phenotype. FBF-1 and FBF-2 promote the
female germ cell fate and are critical for the sperm-to-oocyte switch in the hermaphrodite, functioning to repress the expression of sex determination gene fem-3 (Zhang et al. 1997). PUF-3 and PUF-11 could also function in repressing fem-3. However, we did not observe suppression of the fbf-1(0) fbf-2(0) masculinized germline following removal of nhl-2 (Fig. 6) and the puf-3(0) puf-11(0) double mutant does not show obvious germ cell masculinization (Haupt et al. 2020). A possible explanation is that PUF-3 and PUF-11 have a minor contribution in promoting the female germ cell fate, relative to a major role for FBF-1 and FBF-2; this is consistent with the weak feminization by nhl-2(0), and consistent with the smaller contribution of PUF-3 and PUF-11, relative to FBF-1 and FBF-2, in promoting the GSC fate (Haupt et al. 2020).

C. elegans TRIM-NHL proteins have numerous developmental functions, and our findings reinforce an emerging picture of TRIM-NHL proteins as context-dependent regulators. The nhl-1, nhl-2, nhl-3, and ncl-1 genes were identified by genetic analysis to encode partially redundant regulators of asymmetric cell division in the embryo (Hyenne et al. 2008). Later work showed that NHL-2 positively regulates miRNA activity in the developing larva (Hammell et al. 2009; Karp and Ambros, 2012) and functions downstream of maternally provided miRNAs in the embryo to promote male development and viability (McJunkin and Ambros, 2017), which is distinct from its function in the male germ cell fate in XX larval development described here. A first report of a germline role for nhl-2 was the finding of several defects in nhl-2(ok818)
animals raised at moderate to high culture temperatures: abnormal meiotic chromosome segregation in developing oocytes, reduced brood size, a mortal germline (at 20 °C, 25 °C, and embryonic lethality (at 23–25 °C) (Davis et al., 2018). Their findings implicate NHL-2 activity as important for small interfering (si) RNA-mediated pathway functions in both germ line and soma. Our analysis of NHL-2 activity, carried out primarily at low-moderate temperatures (at 15–20 °C) to take advantage of sensitized genetic backgrounds, complements this other work.

The temperature-sensitive consequences of reduced/absent NHL-2 activity, cold sensitivity (a feminized germline) and heat sensitivity (e.g., abnormal meiotic chromosome segregation), reveal processes that are temperature dependent, where NHL-2 functions to buffer this temperature dependence. We speculate that temperature-dependence may reflect different requirements for NHL-2 targets under different culture conditions, and/or temperature dependence of redundant factors. Significant differences in molecular phenotypes e.g., mRNA and small (s) RNA abundance, have been reported at 20 ° vs 25 °C (Davis et al., 2018). The effects of hnl-2 loss on siRNA function and siRNA abundance at 20 °C may be particularly relevant to the GSC fate/meiotic developmental phenotypes that we observed. In contrast, gene expression changes relevant for the male germ cell fate in hermaphrodite larvae may only be detected at 15 °C. TRIM-NHL proteins are known to function as RNA-binding proteins that regulate target mRNA activity and as E3 ubiquitin ligases to regulate protein turnover. As examples, mammalian TRIM32 promotes neuronal precursor differentiation by ubiquitinating cMyC (Hillje et al., 2011), and C. elegans LIN-41 promotes germline development by repressing translation of numerous target mRNAs (Spiele et al., 2014a; Tocchini et al., 2014). Current data do not allow us to distinguish whether NHL-2 represses paf-3 and puf-11 expression at the level of translational repression/mRNA instability or protein turnover.

We identified two gene products, CGH-1 and ALG-5, that may act together with NHL-2 to influence the stem cell vs meiotic fate choice. Results supporting this possibility include that loss of each of the three genes enhances the tumorous phenotype of glp-1(ar202gfs) and that loss of hnl-2 or alg-5 suppresses the premature meiotic entry phenotype of glp-1(q224) (cgh-1 was not tested). Furthermore, NHL-2 physically associates with CGH-1, as well as miRNA Argonaute proteins ALG-1 and ALG-2, in co-IPs (Hammell et al., 2009; Davis et al., 2018) (ALG-5 was not tested). Our results do not support a role for core miRNA biogenesis factors in regulating the GSC fate/inhibiting meiotic development, as assayed by enhancement of glp-1(ar202gfs). In support of this negative result, in situ hybridization studies have failed to identify a germline expressed miRNA that accumulates in the progenitor zone (Minogue et al. 2018). Instead, NHL-2, ALG-5, and CGH-1 are positioned to interact with both siRNAs and PIWI-associated (pi) RNAs on P granules (Brown et al., 2017; Davis et al., 2018), and NHL-2 activity influences siRNA and piRNA abundance (Davis et al., 2018). Interestingly, the CSR-1 Argonaute complex, presumably through 22G siRNAs, promotes the GSC fate/hibits meiotic entry (Smardon et al., 2000; She et al., 2009). Possibly, NHL-2, ALG-5, and CGH-1 function to oppose the activity of the CSR-1 complex in this decision. Alternatively, or in addition, NHL-2 and CGH-1 may modulate the GSC versus meiotic fate choice in their capacity as translational regulators independent of small RNAs. CGH-1 localizes to numerous RNP particles in the C. elegans soma and germ line where translation regulation may occur (Navarro et al., 2001; Audhya et al., 2005; Boag et al., 2005; Jud et al., 2008; Noble et al., 2008), and it stabilizes certain maternal mRNAs (Noble et al., 2008; Boag et al., 2005) and represses expression of CED-3/caspase to limit apoptosis in the oogenic germ line (Subasic et al., 2016; Navarro et al., 2001). In summary, we provide evidence that NHL-2 modulates the GSC fate vs meiotic development decision by inhibiting the accumulation of PUF-3 and PUF-11, likely explaining the genetic interactions observed with hnl-2(0), suppression of the glp-1(q224) and flb-1(0): flb-2(0) GSC fate defects, and enhancement of glp-1(ar202gfs) tumorous germ line phenotype. An important future question is under what condition(s) does NHL-2 exert this modulatory activity, for example with age or nutrient status?

Declarations of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2022.08.010.

References


