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RESEARCH ARTICLE

Lifespan Extension and Increased Pumping Rate Accompany Pharyngeal Muscle-Specific Expression of *nfi-1* in *C. elegans*

Elena Lazakovitch,[1,2] John M. Kalb,[3] and Richard M. Gronostajski[1,2]*

Caenorhabditis elegans nfi-1* belongs to the Nuclear Factor I (NFI) family of transcription factors known to regulate metazoan gene expression and development. We showed previously that loss of *nfi-1* in worms results in multiple behavioral defects; slower pharyngeal pumping rate, impaired egg laying, defective motility, and a shortened life span. Here, we generated cell-type specific transgenic worms to determine the cells in which *nfi-1* must be expressed to rescue the pharyngeal pumping defect. Expression of *nfi-1* from the pharyngeal muscle-specific *myo-2* promoter, but not from the F25B3.3 or *myo-3* promoters, rescued the pharyngeal pumping defect of *nfi-1* worms. Surprisingly, *myo-2*-driven *nfi-1* expression also rescued the shortened lifespan of *nfi-1* worms, demonstrating a possible cell-autonomous role of *nfi-1* in pharyngeal muscle for both phenotypes. We propose some relationships between the pharyngeal pumping and lifespan phenotypes and potential mechanisms of *nfi-1* function. *Developmental Dynamics 237:2100–2107, 2008.

Key words: transcription; development, aging; *C. elegans*; pharynx

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INTRODUCTION

The pharynx of *Caenorhabditis elegans* is a complex organ that is essential for food intake and distribution through the digestive tract of the worm (Avery, 1993; Avery and Thomas, 1997). Food is taken into the worm and is moved through the intestine by rapid and rhythmic contractions of the pharynx. Pharyngeal pumping is the coordinated and regulated process by which the pharynx mediates intake and forcing of food and waste products through the worm gut. While the rate of pharyngeal pumping is tightly regulated (Avery and Thomas, 1997) and changes with the availability of food, the precise mechanisms that control pharyngeal pumping rate under different physiological conditions are still largely unknown.

The Nuclear Factor I family of transcription/replication proteins is an animal-specific gene family with four members in all vertebrates (NFIA, NFIB, NFIC, and NFIX), single members in simple multicellular animals including *C. elegans* and *Drosophila*, and no members in plants or single cell eukaryotes (Fletcher et al., 1999; Gronostajski, 2000; Murtagh et al., 2003). This property is shared with a very limited set of other transcription factors (14 total, KOG database; Tatusov et al., 2003), including Ski/Sno genes and LIM domain proteins. A major goal of our laboratory is to determine how the functions of the NFI gene family evolved from a single gene in early metazoans to a four gene family in vertebrates. To this end, we have begun a genetic analysis of NFI function in eukaryotes, including *C. elegans* (das Neves et al., 1999; Steele-Perkins et al., 2003, 2005; Lazakovitch et al., 2005).

We showed previously that loss of

[1]Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York
[2]Developmental Genomics Group, New York State Center of Excellence in Bioinformatics and Life Sciences, Buffalo, New York
[3]Department of Biology, Canisius College, Buffalo, New York
Grant sponsor: NASA; Grant number: NNJ06HA59G.
***Correspondence to: Richard M. Gronostajski, Department of Biochemistry, State University of New York at Buffalo, 140 Farber Hall, 3435 Main St., Buffalo, NY 14214. E-mail: rgron@buffalo.edu**

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nfi-1 in worms resulted in a decreased rate of pharyngeal pumping, impaired egg-laying, altered motility and a shortened lifespan (Lazakovitch et al., 2005). To address in which cells *nfi-1* is needed to alleviate these phenotypes, we expressed a green fluorescent protein (GFP) -tagged NFI-1 protein from several cell-type specific promoters and determined which transgenes rescued the phenotypes. Here, we show that expression of *nfi-1* from the pharyngeal-muscle-specific *myo-2* promoter rescues both the pharyngeal pumping and shortened lifespan defects of *nfi-1* null worms, indicating that both of these defects are due to loss of expression of *nfi-1* in these cells. This is the first demonstration of a possible cell-autonomous role of an NFI transcription factor in metazoans and should facilitate the determination of genetic pathways through which NFI transcription factors regulate development.

RESULTS

Transgenic Expression of *nfi-1-GFP* Rescues the Phenotype of *nfi-1* Null Worms

We showed previously that worms homozygous for a deletion of a region of *nfi-1* exhibited altered motility (Unc), decreased egg-laying (Egl), a reduced rate of pharyngeal pumping and a shortened lifespan (Lazakovitch et al., 2005). Because our *nfi-1::GFP* transgenes were expressed in both embryos and adult worms, we asked whether it was possible to generate a GFP-tagged version of *nfi-1* that could be used to both identify the specific cells in which the NFI-1GFP protein is expressed and to rescue the phenotypes of *nfi-1* null worms. For this purpose, a GFP cassette was fused in frame to the penultimate codon of *nfi-1* and a transgene composed of this translational fusion gene and the 3′ untranslated region (UTR) of *nfi-1* was expressed from the *nfi-1* promoter (Fig. 1A). Transgenic worms were generated by injection of this construct and the pRF4 *rol-6* marker plasmid into *nfi-1* worms or N2 worms and screening for Rol progeny. GFP expression was seen in the nuclei of cells that were previously shown to express either endogenous *nfi-1*, or GFP from an *nfi-1::GFP* transgene, including all nuclei in the posterior bulb of the pharynx and in muscle cells of the anterior bulb (Fig. 1B,C), vulval muscles (Fig. 1D,E), body wall muscle (not shown), and gut cells (Fig 1F,G).

After analysis of the *nfi-1* expression pattern, we tested the GFP-tagged *nfi-1* transgene for rescue of *nfi-1* mutant phenotypes. Expression of the *nfi-1::nfi-1-GFP* transgene in *nfi-1* mutants resulted in a partial rescue of the pharyngeal pumping defect in three of four independent transgenic lines generated (Fig. 2A). Differences in rescue are likely due to differences in transgene expression levels. Pharyngeal pumping rates of 1-day-old *nfi-1* worms are significantly lower than in N2 worms ($P < 0.01$) with a more severe reduction in 2-day-old animals (Fig. 2A). Pumping rates of *nfi-1* mutants expressing the transgene are similar to N2 worms on the first day of adulthood ($P = 0.17$). At day 2, pumping rates of rescued transgenic animals are higher compared with *nfi-1* ($P = 0.0028$), but slightly lower compared with N2 ($P = 0.001$). These data indicate that expression of the *nfi-1::nfi-1-GFP* transgene results in a partial rescue of the pharyngeal pumping defect. The transgene was also expressed in N2 worms as a control and had no effect on pumping rates ($P > 0.01$).

To assess rescue of the Egl phenotype by the *nfi-1::nfi-1-GFP* transgene, the number of animals giving rise to a "bag of worms" phenotype were scored. Two lines showed almost complete rescue (3.2–7% of "bags" in different experiments) of the Egl phenotype (Fig. 2B), and two other transgenic lines showed partial reduction in the Egl phenotype (not shown).

Somewhat surprisingly, the lifespan of *nfi-1* mutants expressing *nfi-1::nfi-1-GFP* transgene was not significantly extended by expression of the transgene, possibly because of insufficient or mosaic expression of the transgene (data not shown). These data indicate that the GFP-tagged NFI-1 protein is capable of phenotype rescue when expressed from the endogenous *nfi-1* *cis*-regulatory elements. The change in pharyngeal pumping rate provides a developmental system affected by loss of *nfi-1* that can be examined for a cell-autonomous role of *nfi-1*.

Transgenic Expression of *nfi-1-GFP* Fusion Protein From Neuronal-Specific and Pharyngeal Muscle-specific Promoters

As described above, *nfi-1* mutant worms have a reduced rate of pharyngeal pumping. Because *nfi-1* is expressed in muscle and nonmuscle pharyngeal cells including neurons, gland, and marginal cells, defects in any of these cell types could affect pharyngeal pumping. In addition, muscle and neuronal cells are also potential targets of *nfi-1* function because previous studies showed that pumping rates can be affected by mutations of genes expressed in these cells (Dent et al., 1997; Davis et al., 1999; Robatzek et al., 2001). We, therefore, performed directed expression of *nfi-1* in pharyngeal muscles and neurons to determine in which cells *nfi-1* is needed for normal pharyngeal pumping.

To test whether *nfi-1* has a cell-autonomous function in pharyngeal muscle cells, we made transgenic worms where the *nfi-1-GFP* fusion cassette was driven by the well-characterized pharyngeal muscle-specific *myo-2* promoter. The construct contains the 1.2-kb *myo-2* promoter from plasmid pPD96.48 (Fire lab), the 4.2-kb *nfi-1* genomic region containing all the coding exons of *nfi-1*, the 0.9-kb GFP cassette as a translational fusion to the last residue of *nfi-1*, and the 1.4-kb 3′UTR of *nfi-1* (Fig. 3A). This vector was co-injected with a *rol-6* marker plasmid into wild-type (WT) worms. The resulting transgenic array was then bred into an *nfi-1* null background. Progeny were screened by single-worm polymerase chain reaction (PCR) for the presence of the *nfi-1* deletion allele and lack of the wild-type *nfi-1* allele. As expected, *nfi-1-GFP* expression was seen only in the nuclei of pharyngeal cells in the two lines obtained (Fig. 3B,C).

Directed expression of *nfi-1* in neurons was achieved using the F25B3.3 promoter, which drives expression in all postmitotic neurons (Brignull et al., 2006). The construct contains the 3.5-kb F25B3.3 promoter amplified by PCR from worm genomic DNA fused to the same *nfi-1-GFP* cassette used in

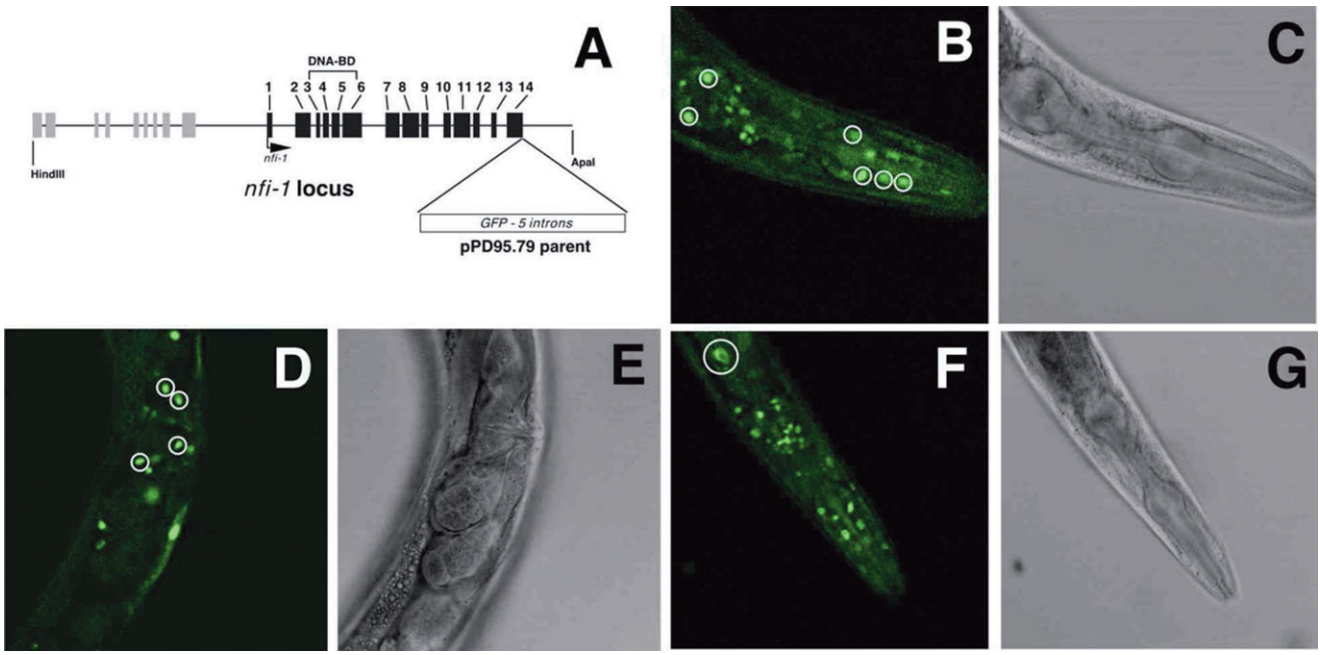

Fig. 1.

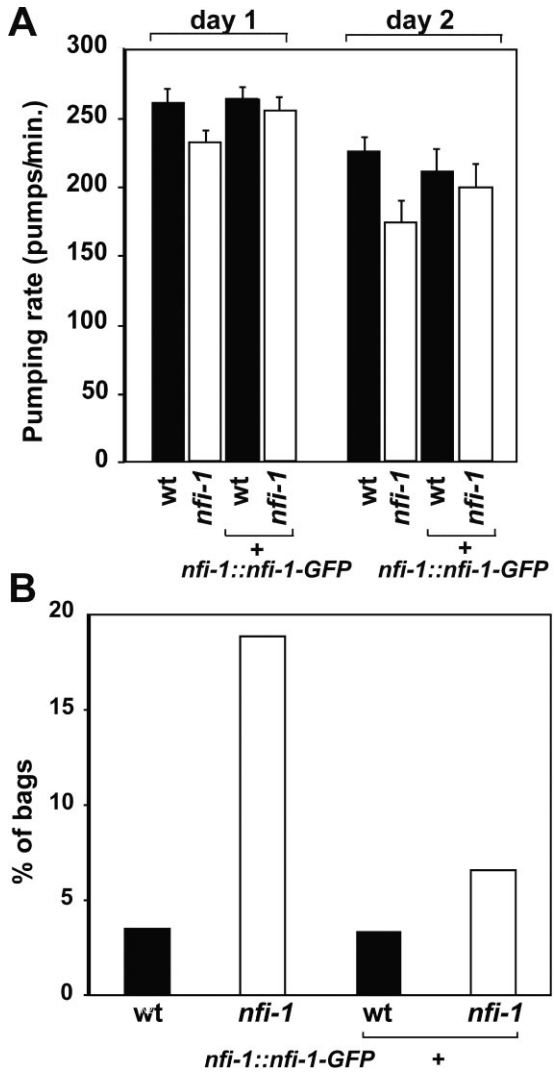

Fig. 2.

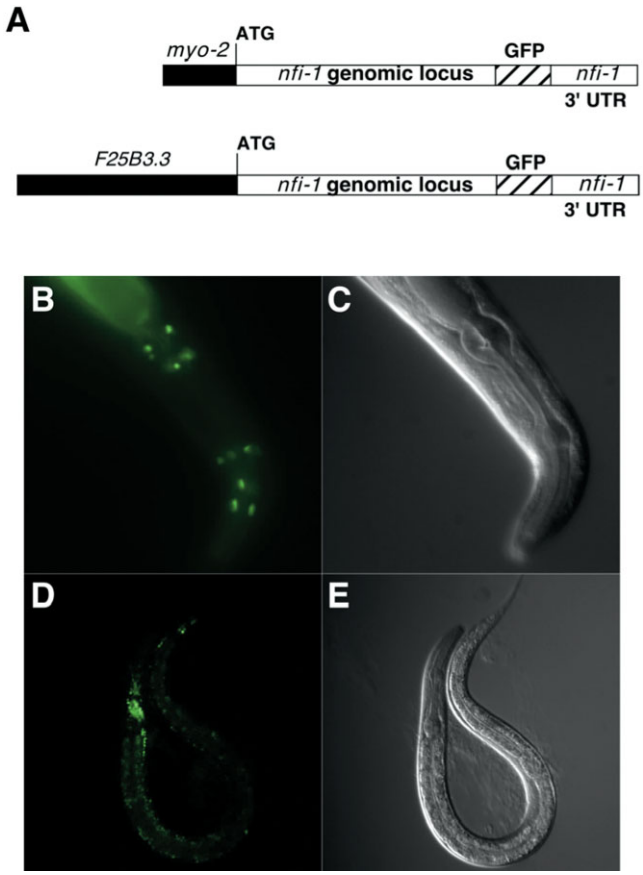

Fig. 3.

the *myo-2::nfi-1-GFP* construct. This vector was coinjected with the *pha-1* rescue plasmid (pBX) into the gonads of *nfi-1;pha-1(ts)* worms and transgenic worms were selected by survival at 25°C. We used the *pha-1* rescue system to ensure transgene expression in all progeny and to eliminate interference by the *rol-6* vector in scoring motility defects. GFP expression was observed as expected in all observed postmitotic neurons of transgenic worms carrying this construct (Fig. 3D,E).

Expression of *nfi-1* in Pharyngeal Muscles Rescues the Pumping and Lifespan Defects of *nfi-1* Mutants, but Expression in Neurons or Body Wall Muscle Does Not

Transgenic expression of *nfi-1* in pharyngeal muscle cells completely rescued the pharyngeal pumping defect of *nfi-1* null worms (Fig. 4A). In addition, the median life span of 17 ± 0.76 days in these transgenic worms was significantly longer than the 11 ± 0.41 days life span of *nfi-1* mutant worms and slightly longer than the 15 ± 0.89 days life span of N2 worms (Fig. 4B). Thus, expression of *nfi-1* from the *myo-2* promoter also rescued the shortened lifespan of *nfi-1* null worms, indicating that expression of *nfi-1* in pharyngeal muscle was responsible for this phenotype (Fig. 4B). However, as expected, the egg-laying phenotype of *nfi-1* mutants was unaffected in these transgenics (Fig. 4C) indicating that this defect is not due to loss of *nfi-1* in pharyngeal muscle.

When *nfi-1* was expressed from the F25B3.3 promoter in neurons of *nfi-1* worms, pharyngeal pumping rates of the transgenic worms were compared with the rates measured in control *pha-1(ts)* and *nfi-1;pha-1(ts)* strains (Fig. 4D). Because of the different growth conditions of these strains (25°C for the transgenic worms and 15°C for *pha-1* and *nfi-1;pha-1(ts)*) worms, all worms were transferred at 20°C for 1 hr before scoring pharyngeal pumping. Pharyngeal pumping rates of the transgenic worms were still reduced compared with *pha-1(ts)* and were similar to the rate measured in *nfi-1;pha-1(ts)* worms (Fig. 4D). Expression of *nfi-1* from the F25B3.3 promoter did not rescue the pumping rate of *nfi-1* mutants. Neuronal expression also did not extend the life span of *nfi-1* mutants (data not shown). Thus, both the pharyngeal pumping and lifespan defects of *nfi-1* null worms were rescued by expression specifically in pharyngeal muscle, but not by expression in all postmitotic neurons.

Lastly, to test whether the motility and egg-laying defects of *nfi-1* null worms were related to loss of *nfi-1* in nonpharyngeal muscle cells, we expressed NFI-1-GFP from the body wall muscle and vulval muscle-specific *myo-3* promoter. A *myo-3::nfi-1-GFP* construct was injected into *pha-1(e2123);nfi-1(qa524)* worms along with *pha-1* rescue plasmid and the resulting transgene was maintained in both *pha-1(e2123);nfi-1(qa524)* and *pha-1(e2123)* backgrounds. Previous studies had shown that *myo-3* is expressed in body-wall and vulval muscles but not pharyngeal muscle (Okkema et al., 1993). As expected, the *myo-3::nfi-1-GFP* transgene was expressed in body wall and vulval muscles (Fig. 5A). However, transgenic expression of *nfi-1-GFP* from the *myo-3* promoter did not rescue the Egl and locomotion defects of *nfi-1* mutants but instead induced a novel phenotype in both *nfi-1* and WT worms. Worms expressing *nfi-1-GFP* from the *myo-3* promoter were partially and progressively paralyzed. Their thrashing rate as young adults was significantly lower compared with all control strains (Fig. 5B). This induced phenotype from *nfi-1* expression in body wall muscle currently makes it impossible to determine whether the cell motility and egg-laying defects in *nfi-1* mutant animals are caused by cell-autonomous defects in body wall muscle.

Pharyngeal-Specific Myosins and *exp-2* Are Down-Regulated in *nfi-1* Mutants

To identify pharyngeal genes affected by loss of *nfi-1*, the transcript levels of more than 30 genes involved in different aspects of pharyngeal pumping were examined by real-time PCR in synchronized adult WT and *nfi-1* worms. Approximately three-fold reductions in the expression of pharyngeal myosins *myo-1*, *myo-2*, and F58G4.1, and in the potassium channel *exp-2* were observed in *nfi-1* worms

Fig. 1. Green fluorescent protein (GFP) -tagged full-length-*nfi-1* transgene (*nfi-1::nfi-1GFP*). **A:** Region of the *nfi-1* locus from the *Hin*dIII site to the termination codon was fused to GFP followed by the *nfi-1* 3′untranslated region (UTR). The plasmid was injected along with pRF4 and RoI progeny selected. **B–G:** Confocal GFP and matching phase contrast images are shown of pharynx (B,C,F,G) and the vulval region (D,E). Expression is seen in all nuclei of the pharynx with nuclei circled in B, from left to right, 2 likely pm7 cells, 1 pm4, 2 pm3s and 1 pm1. Cells circled in D are presumptive vulval muscle cells. Cell circled in F is an intestinal cell.

Fig. 2. A: Rescue of pharyngeal pumping phenotype by the *nfi-1::nfi-1-GFP* transgene. Pharyngeal pumping was counted for 1 min starting on the first day of adulthood for two consecutive days. All animals remained on food during the period of observation. Pharyngeal pumping rates of *nfi-1* worms (white bars) are statistically significantly different from N2 ($P < 0.01$; black bars), with more severe reduction in older vs. younger adult animals. Pumping rates of *nfi-1* mutants expressing the transgene (+*nfi-1::nfi-1-GFP*) are not significantly different from N2 worms on the first day of adulthood ($P = 0.17$). On day 2, the pumping rates of transgenic *nfi-1* worms are significantly higher compared with nontransgenic *nfi-1* ($P = 0.0028$), but slightly lower compared with nontransgenic N2 ($P = 0.001$). The transgene in N2 worms as a control had no significant effect on pumping rates ($P > 0.01$). **B:** Rescue of egg-laying phenotype by the *nfi-1::nfi-1-GFP* transgene. The *nfi-1-GFP* plasmid was coinjected with pRF4 into N2 (black bar) and *nfi-1* null worms (white bar) and RoI progeny selected. The percentage of bagging was scored in N2 (black bar, n = 60), *nfi-1* (white bar, n = 60), *nfi-1::nfi-1-GFP*/N2 (black bar, n = 60), and *nfi-1::nfi-1-GFP/nfi-1* (white bar, n = 45).

Fig. 3. Expression pattern of the *myo-2::nfi-1-GFP* and *F25B3.3::nfi-1-GFP* reporter transgenes. **A:** The structure of the *myo-2::nfi-1-GFP* and *F25B3.3::nfi-1-GFP* fusion constructs are shown. The *myo-2* and F25B3.3 promoter regions are shown in black, white boxes indicate *nfi-1* genomic locus with coding region and 3′untranslated region (UTR). Green fluorescent protein (GFP) is shown as a hatched box. **B,C:** Expression of the *myo-2::nfi-1-GFP* reporter was seen in the nuclei of all pharyngeal-muscle cells as was seen previously for transgenes expressed from the *myo-2* promoter. Nonspecific gut autofluorescence is seen at upper left. C: A Nomarski image of the fluorescent field from panel B showing the pharynx structure. **D,E:** Expression of the *F25B3.3::nfi-1-GFP* reporter was observed as expected in postmitotic neurons. E: The corresponding Nomarski image of D.

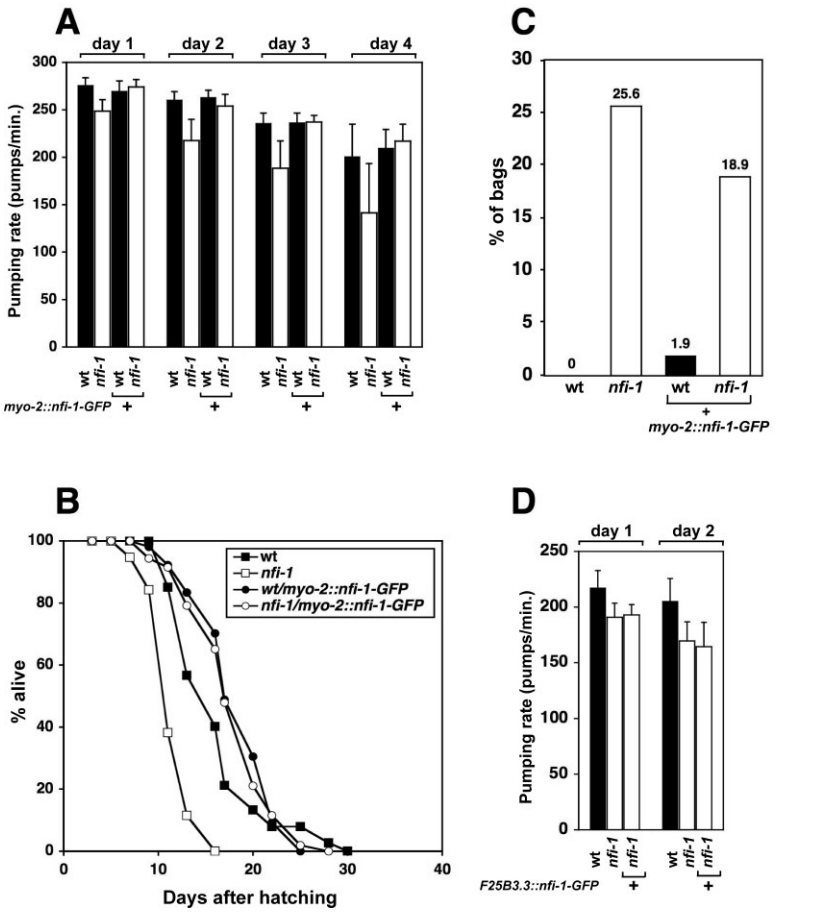

Fig. 4. **A:** Rescue of the pharyngeal pumping defect of *nfi-1* worms by expressing *nfi-1-GFP* from the *myo-2* promoter. Pharyngeal pumping was counted for 1 min starting on the first day of adulthood. All animals remained on food during the period of observation and 15 to 20 worms were scored for each line. Error bars represent a SD. Pharyngeal pumping rates of wild-type (WT) worms and *nfi-1* mutants carrying the *myo-2::nfi-1-GFP* rescue construct are similar to those observed in WT worms while the rate in *nfi-1* worms decreases more severely with time. *pha-1* and *nfi-1;pha-1* worms were cultivated at 15°C and transgenic worms at 25°C until they reach adulthood. Worms were transferred to 20°C for at least 1 hr before scoring pumping rates. Pharyngeal pumping rates observed in *nfi-1* mutants carrying *F25B3.3::nfi-1-GFP* construct are similar to those observed in *nfi-1;pha-1* worms. **B:** Rescue of life span defect of *nfi-1* null worms by expressing *nfi-1-GFP* from the *myo-2* promoter. Survival curves for the strains N2 (n = 48, closed square), *nfi-1* (n = 39, open squares), *myo-2::nfi-1-GFP*/N2 (n = 55, closed circles), and *myo-2::nfi-1-GFP/nfi-1* (n = 83, open circles) are shown. Median life spans for the strains determined by Kaplan-Meier analysis (SPSS software) were 15 ± 0.89, 11 ± 0.41, 17 ± 1.03, 17 ± 0.76 days, respectively. **C:** Egg-laying defect in *myo-2::nfi-1-GFP/nfi-1* transgenic worms. The number of worms with a "bag of worms" phenotype was scored for the strains N2 (black bar, 0% n = 48), *nfi-1* (white bar, 25.6% n = 39), *myo-2::nfi-1-GFP*/N2 (black bar, 1.9% n = 53), and *myo-2::nfi-1-GFP/nfi-1* (white bar, 18.9% n = 37). **D:** Pharyngeal pumping rates in *nfi-1* worms carrying *F25B3.3::nfi-1-GFP* transgene. Pharyngeal pumping rates of *pha-1(ts)* worms (black bars), *pha-1(ts);nfi-1* (white bars), and *pha-1(ts);nfi-1* worms carrying the *F25B3.3::nfi-1-GFP* transgene (white bars + *F25B3.3::nfi-1-GFP*) were compared. No significant difference was seen between the *pha-1(ts);nfi-1* and *pha-1(ts);nfi-1* worms carrying the *F25B3.3::nfi-1-GFP* transgene. GFP, green fluorescent protein.

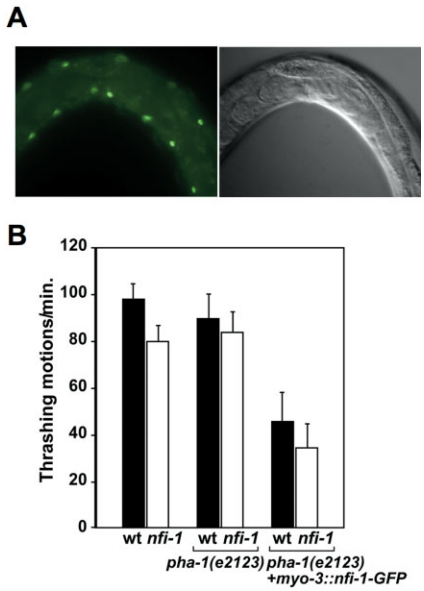

Fig. 5. Expression of *nfi-1-GFP* from the *myo-3* promoter induces motility defects. **A:** The *nfi-1-GFP* transgene was placed under control of the well-characterized *myo-3* promoter. NFI-1-GFP is expressed in the nuclei of body-wall muscles and vulval muscles in *myo-3::nfi-1-GFP* transgenic worms. **B:** Worms expressing NFI-1-GFP from the *myo-3* promoter show a paralyzed locomotion defect. Thrashing rates in M9 media of N2 (black bar) and *nfi-1* (white bar) worms were compared with *pha-1(ts)* (black bar +*pha-1(e2123)*) and *pha-1(ts);nfi-1* (white bar +*pha-1(e2123)*) worms, and *myo-3::nfi-1-GFP* transgene containing *pha-1(ts)* (black bar +*pha-1(e2123)* +*myo-3::nfi-1-GFP*) and *pha-1(ts);nfi-1* (white bar +*pha-1(e2123)*+*myo-3::nfi-1-GFP*) in M9 media were scored as the number of motions per min. Ten worms were scored for each strain.

relative to WT worms (Fig. 6). This reduction is significant and reproducible, but less than the ~11-fold reduction in the muscle-specific titin gene (*ttn-1*) seen previously (Lazakovitch et al., 2005) and used as a positive control in the same experiment. Transcript levels of the body-wall specific *myo-3* gene and an unrelated control gene (C06G1.5) were the same in WT and *nfi-1* animals (Fig. 6).

Because NFI is known to bind with high affinity to the dyad symmetric consensus sequence TTGGC(N5)-GC- CAA (Lazakovitch et al., 2005), we screened for the presence of this sequence in the control regions of the *myo-1*, *myo-2*, *F58G4.1*, and *exp-2* genes. These genes lack any predicted perfect NFI dyad symmetric binding sites. While some mammalian promoters have been shown to contain functional hemi NFI binding sites (a single TTGGC or GCCAA site alone) (Cereghini et al., 1987; Bois-Joyeux and Danan, 1994; Alonso et al., 1996; Gao et al., 1996), the absence of high-affinity dyad symmetric NFI binding sites within the myosin and *exp-2* genes raises the possibility that they may not be direct targets of NFI.

DISCUSSION

We showed previously that loss of *nfi-1* in *C. elegans* results in multiple defects including a reduced rate of pharyngeal pumping, a shortened lifespan, altered motility and defec-

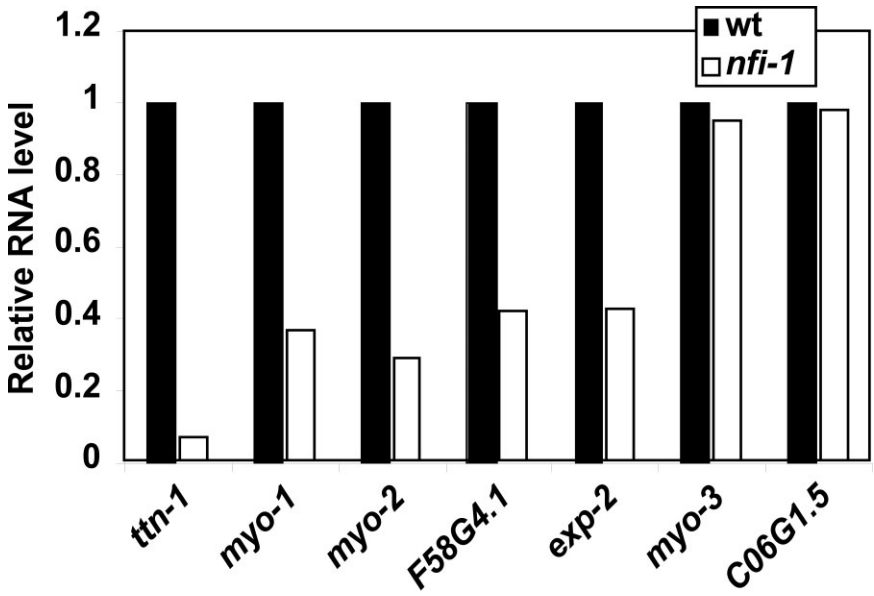

Fig. 6. Changes in transcript levels of pharyngeal myosins *myo-1*, *myo-2*, and F58G4.1 and potassium channel *exp-2* in *nfi-1* mutant worms. RNA from synchronized adult population of wild-type N2 and *nfi-1* worms was extracted and reverse transcribed. The resulting cDNA was analyzed by real-time quantitative PCR (QPCR) using Syber-Green detection in a Bio-Rad iCycler with primers for the indicated genes. Data are presented as transcript levels in *nfi-1* worms normalized to the transcript levels in N2 worms. *ttn-1* was used as a positive control and *myo-3* and C06G1.5 as examples of genes with no changes in expression level. *int-1* was used as a reference gene.

tive egg-laying (Lazakovitch et al., 2005). Here, we have shown that an NFI-1-GFP fusion protein under the control of endogenous *nfi-1* regulatory elements is expressed in multiple cell types in *C. elegans* (Fig. 1) and can partially or fully rescue the pharyngeal pumping and egg-laying defects of *nfi-1* worms (Fig. 2). These data also show that expression of this chimeric NFI-1-GFP fusion protein specifically in pharyngeal muscle cells (Fig. 3) rescues both the pharyngeal pumping rate defect and the shortened lifespan of *nfi-1* worms (Fig. 4). Thus, it appears that both the altered pharyngeal pumping rate and reduced lifespan reflect a cell-autonomous role for *nfi-1* in pharyngeal muscle cells.

Perhaps the most surprising finding seen here is that the reduced lifespan of *nfi-1* null worms is rescued by *nfi-1* expression in pharyngeal muscle cells (Fig. 4). These data indicate that lifespan may be directly linked to alterations in pharyngeal pumping rate. While cause and effect cannot be directly inferred from these data, the correlation between pharyngeal pumping rescue and lifespan extension is striking and warrants further investigation. Previous studies indicated that a reduced rate of pharyngeal pumping was highly correlated to organismal aging in *C. elegans* (Huang et al., 2004). Pumping rates decrease with age in wild-type animals and mutations that affect lifespan concomitantly affect pumping rates (Huang et al., 2004). This strong correlation between reduced rates of pharyngeal pumping and accelerated aging appears initially to be paradoxical, because caloric restriction is well know to extend lifespan in several organisms (Koubova and Guarente, 2003). Indeed in *C. elegans*, some mutations in *eat-2* which reduce overall pharyngeal pumping rate can extend adult lifespan. However, such apparent discrepancies appear to be resolved when the pumping rate is integrated over the adult lifespan of worms, when it becomes clear that decreased pumping correlates with reduced lifespan (Huang et al., 2004). A major unanswered question is the mechanistic connection between pumping rate and lifespan; does reduced pumping directly result in shorter lifespan, are the two phenotypes independently controlled by a common regulatory mechanism, or is the apparent correlation between the two phenotypes merely coincidental? Our data represent independent confirmation of this apparent connection between pharyngeal pumping rate and lifespan in *C. elegans*.

The control of pharyngeal pumping rate is a complex process that involves both the pharyngeal muscle cells and neurons that innervate the pharynx (Avery and Thomas, 1997). Early laser ablation studies showed that while none of the 20 neurons that innervate the pharynx are essential for pumping, loss of the M4, MC, and M3 neurons affect the response of the pharynx to feeding cues (Avery and Horvitz, 1989; Raizen et al., 1995). The cellular, biochemical and genetic pathways that control the pumping rate in response to food are manifold and under active investigation. Mutations in several genes including *egl-30*, *eat-10*, and *eat-11* (Robatzek et al., 2001; Patikoglou and Koelle, 2002) affect pharyngeal pumping rate. These 3 genes all encode proteins involved in G-coupled signaling networks, one pathway known to affect pumping rates. While we described previously that the transcript levels of these genes are unaffected in *nfi-1* null worms, it is possible that signaling through this system is affected by loss of *nfi-1*. Because some of the *eat* genes are known to be expressed only in neurons, the cell-autonomous rescue of pumping by *nfi-1* expression solely in pharyngeal muscles narrows our focus to genes functioning in these cells.

Because *nfi-1* encodes a transcription factor, it will be essential to discover the downstream target genes regulated by NFI-1 that are ultimately responsible for the phenotypes seen in the *nfi-1* null worms. The identification of pharyngeal muscle cells as the likely mediator of the pharyngeal pumping defect is an important first step in this process. Nonetheless, our initial efforts at identifying direct target genes for NFI-1 have been so far unfulfilled. We have identified several genes, in addition to the previously identified *ttn-1* (Lazakovitch et al., 2005). whose expression is reduced in *nfi-1* null worms, including *myo-1*, *myo-2*, *F58G4.1*, and *exp-2* (Fig. 6). At least two of these genes, *myo-1* and *myo-2* are expressed in pharyngeal muscle and could be direct targets of *nfi-1* transcriptional activation in

these cells. However, the promoter regions of these genes do not contain strong predicted NFI binding sites; thus, it is possible that they are indirect rather than direct targets of NFI-1.

It remains to be determined the precise mechanism(s) by which NFI-1 affects pharyngeal pumping rate and lifespan. Because NFI-1 is a transcription factor, the simplest mechanism would be through direct regulation of genes in pharyngeal muscle cells that are required for pumping. Whether such phenotype-proximal genes are direct transcriptional targets of NFI-1, or indirect targets whose levels or activity is affected by the expression level of direct NFI-1 target genes, is unknown. Because NFI-1 expression solely in pharyngeal muscle cells is sufficient to rescue the pumping and lifespan defects, our working hypothesis is that NFI-1 is functioning in a cell-autonomous manner in these cells. However, other models are clearly possible. For example, rescue of the phenotypes could occur through a reciprocal-inductive process by which NFI-1 would directly or indirectly regulate specific genes in pharyngeal muscle cells, and the products of these genes would affect the function of neighboring cells through some paracrine inductive process (i.e., acting as hormones, soluble growth factors, cell surface molecules, etc.) and these neighboring cells would then reciprocally feed back on pharyngeal muscle cells to influence the phenotypes. It will require (1) identification of the direct NFI-1 target genes in pharyngeal muscle cells, (2) discovery of possible indirect downstream targets of NFI-1 in these cells, and (3) the characterization of the phenotype-proximal genes that mediate the phenotype of *nfi-1* worms, to determine the mechanism(s) by which NFI-1 affects pharyngeal pumping and lifespan.

EXPERIMENTAL PROCEDURES

Strains and Transgene Plasmids

The Bristol strain N2 was used as wild-type (WT) and worms were grown at 20°C using standard techniques (Wood, 1988). Strain GE24 *pha-1(2123)* III and the pBX rescue plasmid for the injection (Granato et al., 1994) were kindly provided by Denise Ferkey (SUNY at Buffalo, NY). pPD95.79, pPD96.48, and pPD96.52 (kindly provided by A. Fire) plasmids were used to generate a translational fusion of *nfi-1* and GFP driven by the native *nfi-1* promoter, the *myo-2* promoter and the *myo-3* promoter, respectively. To generate *nfi-1::nfi-1-GFP* the 3′UTR fragment of *unc-54* in pPD95.79 was replaced at the *Eco*RI and *Eag*I sites with a PCR-amplified fragment containing the 3′UTR of *nfi-1* (chromosome II:7542464-7543880, WormBase WS187). The 5′end of the construct was extended with a fragment (chromosome II:7533993-7542460, WormBase WS187) containing ~4.4 kb of the promoter region and the coding region of *nfi-1*, but lacking a translation stop codon, between *Hin*dIII and *Xma*I sites.

To generate the *myo-2::nfi-1-GFP* and *myo-3::nfi-1-GFP* expression vectors a unique *Nhe*I site was generated by PCR immediately upstream of the ATG translation initiation codon of *nfi-1* in the *nfi-1::nfi-1-GFP* vector. An *Nhe*I-*Eag*I fragment of this plasmid containing the coding region for the *nfi-1-GFP* fusion protein was cloned into corresponding sites of pPD96.48 and pPD96.52 to produce *myo-2::nfi-1-GFP* and *myo-3::nfi-1-GFP,* respectively. To generate the *F25B3.3::nfi-1-GFP* vector a 3.5 kb promoter region of *rgef-1* (chromosome V:9561563-9565134, WormBase WS187) was PCR amplified from *C. elegans* genomic DNA using primers containing *Bam*HI and *Nhe*I restriction sites and cloned into the corresponding sites of *myo-2::nfi-1-GFP* to replace the *myo-2* promoter.

The construct *nfi-1::nfi-1-GFP* (25 ng/μl) was injected into the wild-type N2 and *nfi-1* deletion strain XA549 *nfi-1(qa524)*II along with *rol-6(gf)* marker plasmid pRF4 (175 ng/μl) using standard microinjection procedures (Wood, 1988). The construct *myo-2::nfi-1-GFP* (1 ng/μl) was injected into N2 worms along with plasmid pRF4 (175 ng/μl). The resulting transgenic arrays were crossed onto the *nfi-1* deletion strain XA549 *nfi-1(qa524)*II. A double mutant strain *pha-1(e2123);nfi-1(qa524)* was made for analysis of locomotion defects. The constructs *myo-3::nfi-1-GFP* (10 ng/μl) and *F25B3.3::nfi-1-GFP* (10 ng/μl) were injected into *pha-1(e2123);nfi-1(qa524)* strain along with *pha-1* rescue plasmid pBX (120 ng/μl) and plasmid pBluescript (70 ng/μl). The resulting transgenic array for *myo-3::nfi-1-GFP* construct was crossed onto the *pha-1(e2123)* strain containing the wild-type *nfi-1* allele.

Behavioral and Functional Assays

Wild-type N2 and *nfi-1* mutant transgenic worms carrying the *rol-6(gf)* marker were reared at 20°C, transgenic worms carrying *pha-1(e2123)*III mutation at 25°C, and GE24 *pha-1(e2123)*III strain at 15°C on NGM/OP50 plates. To produce age-synchronized worms, young adults were placed on a bacterial lawn and allowed to lay eggs for 3 hr. Pharyngeal pumping was counted for 1 min starting on the first day of adulthood. Worms were placed on NGM/OP50 plates at 20°C and left undisturbed for 1 hr before measuring. All animals remained on food during the period of observation. “Bag of worms” phenotype was scored for each strain using age-synchronized worms. Worms were observed each day from the start of egg laying until 1 day after cessation of egg laying and were moved to new plates every second day to prevent overcrowding and starvation. For life-span assays, worms were maintained at 20°C and adult worms were moved to new plates every second day until progeny production ceased. Worms were observed every day and were scored as dead when they failed to respond to touch. Animals that crawled off the plate or died from internally hatched progeny were censored, but incorporated into the data until the day of disqualification. For scoring locomotion rate, worms were placed on NGM/OP50 plates at 20°C for 1 hr before measuring. Each worm was placed in a 100 μl drop of M9 buffer (Brenner, 1974), and thrashing motions were observed under a dissecting microscope and counted for 1 min.

RNA Purification and Quantitative PCR

Synchronized populations of WT and *nfi-1* mutant worms were generated by hatching eggs after alkaline hypochlorite-treatment. The worms were collected as gravid adults. Total RNA was prepared with Trizol (Life Technologies). cDNA was synthesized using random primers and Super-Script First-Strand Synthesis System (Invitrogen). Real-time quantitative PCR (QPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) on a Bio-Rad iCycler. The *inf-1* gene was used as an internal control for RT-PCR reactions and to normalize quantifications in QPCR reactions. Primers used in QPCR are available on request.

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