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The CCAAT-box transcription factor, NF-Y complex, mediates the specification of the IL1 neurons in *C. elegans*

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ABSTRACT

Neuronal differentiation is highly coordinated through a cascade of gene expression, mediated via interactions between *trans*-acting transcription factors and *cis*-regulatory elements of their target genes. However, the mechanisms of transcriptional regulation that determine neuronal cell-fate are not fully understood. Here, we show that the nuclear transcription factor Y (NF-Y) subunit, NFYA-1, is necessary and sufficient to express the *flp-3* neuropeptide gene in the IL1 neurons of *C. elegans*. *flp-3* expression is decreased in dorsal and lateral, but not ventral IL1s of *nfya-1* mutants. The expression of another terminally differentiated gene, *eat-4* vesicular glutamate transporter, is abolished, whereas the *unc-8* DEG/ENaC gene and pan-neuronal genes are expressed normally in IL1s of *nfya-1* mutants. *nfya-1* is expressed in and acts in IL1s to regulate *flp-3* and *eat-4* expression. Ectopic expression of NFYA-1 drives the expression of *flp-3* gene in other cell-types. Promoter analysis of IL1-expressed genes results in the identification of several *cis*-regulatory motifs which are necessary for IL1 expression, including a putative CCAAT-box located in the *flp-3* promoter that NFYA-1 directly interacts with. NFYA-1 and NFYA-2, together with NFYB-1 and NFYC-1, exhibit partly or fully redundant roles in the regulation of *flp-3* or *unc-8* expression, respectively. Taken together, our data indicate that the NF-Y complex regulates neuronal subtype-specification via regulating a set of terminal-differentiation genes.

INTRODUCTION

The proper function of animal nervous systems must accurately execute developmental programs that generate and specify neuronal cells and circuits to properly form and function. These processes allow neuronal cells to acquire the correct temporal and spatial identity and cell-type-specific functions. Dysregulation of these programs leads to multiple neuro-developmental and neuro-psychiatric diseases (1). Although it has been well established that transcriptional control via *trans*- and *cis*-acting regulatory networks mediate neuronal differentiation and specification (2, 3), the molecular and neuronal mechanisms underlying transcription-mediated neuronal development are still not completely understood.

Caenorhabditis elegans has a relatively simple nervous system with 302 neurons categorized into 118 morphologically distinct classes (4, 5). Developmental programs to generate and specify these neurons have been well-characterized; distinct transcription regulatory networks result in cell-specific expression of pan-neuronal and terminal features such as synaptic vesicle components and neurotransmitters, respectively (3, 6-8). Hundreds of *C. elegans* transcription factors, including 102 homeobox genes, play roles in cell-fate determination, and many of them provide necessary and sufficient conditions to specify neuronal identity by acting as terminal selectors, which directly or indirectly regulate the expression of most terminal differentiation genes (2, 3, 9). However, the complete molecular mechanisms of how individual neurons achieve cell-type specific determination and specification are still not understood.

C. elegans inner labial neuron type 1 (IL1) multimodal sensory/inter/motor neurons. Three pairs of radially symmetric neurons, including two dorsal (IL1 dorsal left [IL1DL] and right [IL1DR]), two lateral (IL1 lateral left [IL1L] and right [IL1R]), and two ventral pairs of neurons (IL1 ventral left [IL1VL] and right [IL1VR]). IL1s innervate the head muscles with

their cell bodies located in the head (5, 10, 11) (Fig.1A). Their processes run anteriorly to the tip of the nose, and their function may play roles in head locomotion (12). Several *trans*-acting factors are expressed in IL1s, including homeodomain (*ceh-43/Dlx*, *ceh-32/Six3*, *ceh-18*/POU, *vab-3/Pax6*, and *zfh-2*/zinc-finger protein 2) and non-homeodomain (*lin-32/Atonal* bHLH and *sox-2/SoxB1* HMG) types of transcription factors (5, 8, 13-15). The *lin-32* gene regulates both pan-neuronal and terminal differentiation via the *ceh-43* and *ceh-32* homeobox genes, which are also regulated by *sox-2* and *vab-3* genes (13-15). However, transcriptional regulation underlying the IL1 neuronal specification needs to be further understood.

RESULTS

Expression of a neuropeptide gene in the lateral and dorsal neurons of IL1 is abolished in *nfya-1* mutants

To identify factors that specify the neuronal cell-fate of IL1s, we performed a non-biased genetic screen to isolate mutants. The expression pattern of a *flp-3*p*::gfp* reporter was disrupted in IL1s (Fig.1B). *flp-3* encodes an FMRFamide-related neuropeptide and is expressed in all six IL1 neurons as adult (16). Among mutants isolated from this screen, one mutant allele (*lsk53*) showed weak expression of *flp-3*, specifically in the lateral and dorsal IL1s, but the relatively normal expression in the ventral IL1s (Fig.1C, D and Supplementary Fig.1). We quantitated the expression phenotype of the *flp-3* gene in IL1s of *lsk53* mutants and found that *lsk53* mutants showed significantly lower *flp-3* expression in the lateral and dorsal IL1 neurons than wild-type animals. The average intensity for *lsk53* mutants (IL1L: 50.03±13.98 A.U., n=50; IL1R: 58.06±11.36 A.U., n=50) was only about 38% (IL1L) or 46% (IL1R) of that of wild-type animals (IL1L: 132.32±9.36 A.U., n=50; IL1R: 127.08±4.56 A.U., n=50). The *flp-3* expression in dorsal IL1 of *lsk53* was also significantly reduced; however,

the *flp-3*p*::gfp* intensity in the ventral IL1s was comparable to that of wild-type animals (Fig. 1D).

From the genetic mapping and whole-genome sequence analysis, we identified a molecular lesion of *lsk53* mutant allele in the *nfya-1* gene (Fig.1E). *nfya-1* encodes a NF-YA subunit that constitutes the NF-Y trimer complex with NF-YB and NF-YC subunits and has been shown to regulate the transcription of the *egl-5* and *tbx-2* transcription factors (17-19). *nfya-1* has a conserved CCAAT-binding factor (CBF) domain which is highly conserved through evolution (20) (Fig.1E). The *lsk53* mutant allele carries a nonsense mutation in the second exon that results in a premature translation stop (R117*Opal*), suggesting that this mutation is a null allele (Fig.1E). Then we tested an additional null allele (*ok1174*) of *nfya-1* (21), in which the expression of *flp-3* was decreased similarly to the *lsk53* allele (Fig.1F). These findings indicate that NFYA-1 plays a role in regulating *flp-3* neuropeptide gene expression in the lateral and dorsal, but not ventral IL1 neurons. It is noteworthy that we had not observed ectopic *flp-3* expression in other cell-types (Supplementary Fig.2). This result indicates that *nfya-1* may act as a positive regulator, in contrast to its negative role for *egl-5* or *tbx-2* expression (18, 19).

Expression of the IL1-specific marker genes is differentially affected in *nfya-1* mutants

The IL1 neurons are both peptidergic and glutamatergic (16, 22). To determine the extent to which *nfya-1* regulates gene expression in IL1s, we assessed the expression of IL1-specific terminal differentiation genes, such as the glutamatergic marker genes. We tested *eat-4* vesicular glutamate transporter and *unc-8* DEG/ENaC cation channel protein, of which reporter genes are expressed in IL1s (22, 23). We first confirmed that *eat-4* (481bp sequence located downstream of the translation start site: *eat-4*p*12*) (22) and *unc-8* (293bp sequence

located ~197bp upstream of the translation start site: *unc-8Δ7*p) were indeed expressed in all six IL1 neurons (Supplementary Fig.3). We next examined the expression pattern of *eat-4* and *unc-8* in *nfya-1* mutants. The expression of *eat-4* was abolished in all six IL1 neurons, whereas that of *unc-8* was not affected (Fig.1G, H and Supplementary Fig.4), suggesting that *nfya-1* differentially regulates gene expression of terminal differentiation markers in the IL1 neurons.

Next, to test whether other neuronal characteristics are retained in IL1s of *nfya-1* mutants, we examined the expression of the pan-neuronal gene markers, *rgef-1* Ras guanine nucleotide releasing protein and *unc-119* chaperone (24, 25) (Fig.1I, J and Supplementary Fig.3, 4), and found that the GFP expression of the *rgef-1*p*::gfp* and *unc-119*p*::gfp* reporters was not changed in IL1s of *nfya-1* mutants, confirming that *nfya-1* regulates the expression of specific and limited genes, including *flp-3* and *eat-4*, in IL1s.

The IL2 (inner labial neuron type 2) neurons, which are also embedded in the inner labial sensilla, are structurally similar to IL1s and closely related in cell lineage, where the sisters of the IL1 precursor cells are the IL2 neurons (4) (Supplementary Fig.5). To examine whether *nfya-1* also affects the characteristics of IL2, we tested the expression pattern of the *tba-6* α-tubulin gene, an IL2-specific marker gene (26) in *nfya-1* mutants, and found that the expression of *tba-6* was not altered in IL2s of *nfya-1* mutants (Supplementary Fig.5), indicating that *nfya-1* may play specific roles in the differentiation of IL1s.

nfya-1 is expressed and functions in the IL1 neurons to regulate _flp-3_ and _eat-4_ expression

nfya-1 has previously been expressed in most cell-types, including neuronal,

intestinal, and germline cells of developing larvae and adults (17, 18). To determine whether *nfya-1* is expressed in the IL1 neurons, we generated a transgenic animal expressing the *nfya-1*p*::gfp* transgene that includes 2.1kb of an upstream sequence of the *nfya-1* gene (Fig.1E) and examined its expression pattern (Fig.2A and Supplementary Fig.6A, B). Consistent with previous reports, *nfya-1* was expressed in many neurons in the head of worms (17, 18). To identify *nfya-1* expression in IL1s, we generated a transgenic line expressing the *nfya-1*p*::gfp* transgene together with the *flp-3*p*::mCherry* transgene. We found that *nfya-1* was co-expressed with *flp-3* in all IL1s at either the adult or larval developmental stage animals, indicating that *nfya-1* is indeed expressed in IL1s throughout development (Fig.2A and Supplementary Fig.6A, B). Moreover, GFP-tagged NFYA-1 driven under the control of the *nfya-1* promoter was localized to the nucleus of all IL1 neurons, supporting its predicted role as a transcription factor (Fig.2B and Supplementary Fig.6C).

To determine whether *nfya-1* acts cell-autonomously within IL1, we expressed a wild-type *nfya-1* cDNA under the control of the *nfya-1* promoter or *unc-8Δ7* IL1-specific promoter in *nfya-1* mutant backgrounds. The *nfya-1* expression from these transgenes fully rescued the *flp-3* expression defects in IL1s of *nfya-1* mutants (Fig.2C and Supplementary Fig.7), indicating that *nfya-1* acts in IL1s to regulate *flp-3* expression. Moreover, defects in the *eat-4* expression of *nfya-1* mutants were also restored by the *nfya-1* expression under the control of *nfya-1* promoter as well as IL1-specific promoter (Fig.2D). Together, *nfya-1* is expressed in and acts in the IL1 neurons to regulate the expression of a subset of IL1 marker genes.

Expression of *nfya-1* is partially sufficient to induce *flp-3* expression in other cell-types

To address when *nfya-1* is required for *flp-3* expression in the IL1 neurons, we tried

to trigger a temporal *nfya-1* expression under the control of an inducible, ubiquitously expressed heat-shock promoter (*hsp16.2*) (27). Although we tried to heat-shock animals in several contexts, temporal expression of the *nfya-1* gene at the egg, larval, or adult stage did not rescue the defects in *nfya-1* mutants, suggesting that acute, post-developmental expression of *nfya-1* does not induce appropriate *flp-3* expression in IL1s (Fig.2E).

To further investigate whether the expression of *nfya-1* can induce *flp-3* expression in other cell-types, we forced *nfya-1* expression under the control of the *flp-7* neuropeptide promoter, which is normally expressed in several head neurons, including a I5 pharyngeal neuron, but not in IL1s (16, 28, 29) (Fig.2F). We found that *nfya-1* expression under the control of the *flp-7* promoter did not affect *flp-3* expression in IL1s but induced ectopic *flp-3* expression in the I5 neuron in about 11% of the transgenic worms (Fig.2F), indicating that expression of NFYA-1 can induce *flp*-3 expression in other cells that do not relate to IL1. These results suggest that ectopically-expressed *nfya-1* can drive the expression of the IL1 marker in non-IL1 cells.

Identification of *cis*-regulatory motifs of terminally differentiated IL1 markers

To identify *cis*-regulatory motifs required to drive the expression of the IL1 marker genes, *flp-3, eat-4,* and *unc-8,* in the IL1 neurons, we performed a promoter analysis in which DNA sequences within the promoters were serially deleted, and the resultant transgenic animals were examined for expression in IL1s. We found that deletions in several regions of the *flp-3* promoter caused decreased *flp-3* expression in IL1s; a 101bp sequence between *Δ3* and *Δ4,* a 27bp sequence between *Δ4* and *Δ5,* and a 78bp sequence between *Δ5* and *Δ6* (Fig.3A). We tried further to narrow down the regions by point-mutating DNA sequences and identified two DNA sequences or motifs; CCTCAATTTAT for *flp-3* expression in the lateral

IL1s and CCCAACACTCCTT for *flp-3* expression in the dorsal and ventral IL1s (Fig.3A). The lateral IL1 motif appears to be phylogenetically conserved in the promoters of the *flp-3* orthologs of related *Caenorhabditis* species and shares core sequences with the mammalian NF-YA binding motif (CCAAT). However, the dorsal and ventral motif was not found in the *flp-3* promoters of related *Caenorhabditis* species and did not contain an NF-YA binding motif (Fig.3A).

Additionally, we analyzed the *eat-4* and *unc-8* gene promoters. Previously, a minimal region within the *eat-4* promoter for *eat-4* expression in IL1s was identified (22). We further mutated DNA sequences in this region which are highly conserved within related *Caenorhabditis* species. We identified the DNA sequence (AAGGCACACGGCCGTGA), and mutations in this sequence resulted in an almost complete loss of *eat-4* expression in all IL1s (Fig.3B). We next identified a minimal region in the *unc-8* promoter by serially deleting DNA sequences and mutated conserved DNA sequences within a 293bp sequence between *Δ5* and *Δ6* (Fig.3C). This four bases (GGAA) DNA sequence is crucial to the expression of the *unc-8* gene in all IL1s. We noted that these DNA sequences for the IL1 expression of *eat-4* and *unc-8* appeared unrelated to the conventional mammalian NF-Y binding motif of CCAAT (Fig.3B, C). Taken together, these results suggest that distinct *cis*-regulatory motifs play roles in expressing the terminally expressed IL1 genes.

To test whether these IL1L/R motifs act to drive gene expression in the IL1 neurons, we inserted three copies of the IL1L/R motifs in the promoter of *flp-7* (28) (Supplementary Fig. 8). Transgenic animals expressing a *flp-7*p-IL1L/R *motif::gfp* reporter construct still exhibited GFP expression in *flp-7* expressing neurons, and we observed ectopic expression of *flp-7* in the head. This result indicates that the insertion of IL1L/R motif into the *flp-7* promoter does not drive a reporter in IL1s, but does in other cells, which do not usually

express *flp-7*. These results suggest that inserted motifs may change *flp-7* promoter activity to drive gene expression in other cells, possibly by interacting with NF-Y.

Sequence similarity between the lateral IL1 motif of the *flp-3* gene promoter and the mammalian NF-Y binding motif prompted us to test whether the *C. elegans* NFYA-1 can directly bind this DNA sequence in the *flp-3* gene promoter *in vivo*. Previously, the *C. elegans* NF-Y complex has been shown to bind directly to a CCAAT site identified in the *egl-5* gene promoter via an electrophoretic mobility shift assay (EMSA) (18). We performed a chromatin immunoprecipitation (ChIP) assay coupled to a quantitative real-time PCR using an antibody specific to GFP and extracts derived from transgenic animals expressing the *nfya-1*p*::gfp* (GFP only) or *nfya-1*p*::*NFYA-1*::gfp* (GFP-tagged NFYA-1) transgenes. We selected five loci, including the *flp-3, egl-5, eat-4, unc-8,* and *flp-12* genes. The *flp-12* gene promoter, which is expressed in the SMB neuron specifically, was not altered in *nfya-1* mutants, served as a negative control (16, 28) (Supplementary Fig.9). We found significant enrichment of *flp-3* fragments but not *egl-5, eat-4, unc-8,* or *flp-12* fragments in extracts from transgenic animals expressing NFYA-1-GFP (Fig.3D), indicating that NFYA-1 can directly bind to the CAAT sequences identified in the *flp-3* promoter sequences.

nfya-1 and _nfya-2_ function redundantly, with _nfyb-1_ and _nfyc-1_, to regulate the expression of the IL1 markers

In contrast to the mammal, which has a single NF-YA gene, the *C. elegans* has two *nfya* genes, *nfya-1* and *nfya-2* (17, 18). These two *nfya* genes share similar DNA sequences and expression patterns. However, it has been reported that only *nfya-1*, but not *nfya-2*, regulates *egl-5* and *tbx-2* repression, and this result has raised questions about the roles of *nfya-2* (17, 19). Therefore, we examined whether *nfya-2* regulates the gene expression of IL1

markers and found that *flp-3* expression was affected in *nfya-2(tm4194)* mutants, in a similar manner observed in *nfya-1* mutants (Fig.4A and Supplementary Fig.10); *flp-3* expression was significantly decreased in the dorsal and lateral IL1s but not ventral IL1s (Fig.4A, B). The *flp-3* expression in *nfya-2;nfya-1* double mutants was decreased even in the ventral IL1s, whose *flp-3* expression was not affected in any single mutant background of *nfya* (Fig.4A, C). Moreover, the *flp-3* expression in all six IL1s was decreased in NF-YB *nfyb-1(cu13)*, or NF-YC *nfyc-1(tm4541)* mutants, (Fig.4A, B and Supplementary Fig.10). These data indicate that *nfya-1* and *nfya-2* act in a partially redundant fashion together with other NF-Y components, *nfyb-1* and *nfyc-1*, to regulate *flp-3* expression in all IL1s.

Gene expression of *eat-4* was decreased in IL1s of *nfya-1* mutants, but not that of *unc-8*. Interestingly, the *nfya-2* mutation did not affect gene expression of either *eat-4* or *unc-8* in IL1s (Fig.4D, E and Supplementary Fig.11). However, the *unc-8* expression was strongly compromised in *nfya-2;nfya-1* double mutants, indicating the redundant functions of *nfya-1* and *nfya-2* in regulating *unc-8* expression in IL1s. In addition, the *eat-4* and *unc-8* expressions were significantly decreased in *nfyb-1* or *nfyc-1* mutants (Fig.4D, E). These data, together with the *flp-3* expression results, indicate that the NF-Y complex plays distinct roles in regulating IL1 marker expression.

nfya-2, *nfyb-1*, and *nfyc-1* have been previously shown to be expressed in many head neurons in adults (17, 18). We generated transgenic animals expressing *gfp* under the control of a 1.2kb sequence upstream of *nfya-2*, 2kb sequence upstream of *nfyb-1*, or 495bp sequence upstream of *nfyc-1* (Supplementary Fig.10) and examined their expression pattern. Although our *nfya-2*p*::gfp* or *nfyc-1*p*::gfp* transgenic animals did not exhibit any detectable *gfp* expression, the *nfyb-1*p*::gfp* transgenic animals showed strong *gfp* expression in all six IL1s (Fig.4F), supporting that the NF-Y complex acts in IL1s to regulate IL1 marker expression.

Due to the evolutionary conservation of NF-Y, understanding the molecular and neuronal mechanisms of the highly conserved NF-Y complex underlying neuronal specification in *C. elegans* will help understand what the function of NF-Y is in cell differentiation, which is the pivotal process in developmental of multicellular organisms (30).

DISCUSSION

See supplementary information for Discussion

MATERIALS AND METHODS

See supplementary information for Materials and Methods

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

FIGURE LEGENDS

Figure 1. *nfya-1* regulates expression of the terminally expressed markers in the IL1 neurons. (A) Schematic drawing of the IL1 neurons in *C. elegans*. (B) Experimental scheme of EMS mutagenesis. (C) Representative images of wild-type and *nfya-1* mutants (*lsk53* and *ok1174*) expressing the *flp-3*p*::gfp* transgene in IL1s. Anterior is to the left. Scale bar=20μm. (D) Relative levels of *flp-3*p*::gfp* fluorescence in IL1s of wild-type and *lsk53* mutants. N=5 for each (ten worms per N). (E) Genomic structure of the *nfya-1*gene. The gray lines indicate the promoter region of *nfya-1* fused to the *gfp* gene, respectively. (F) Relative levels of *flp-3*p*::gfp* fluorescence in each IL1 cell of wild-type and *ok1174* mutants. N=3 for each. (G) Percent of animals expressing the *eat-4*p*12::gfp* transgene in IL1s. n=30–50 for each. (H-J) Representative level of wild-type and *nfya-1* mutants expressing the *unc-Δ7*p*::gfp* (H), *rgef-1*p*::gfp* (I), *or unc-119*p*::gfp* (J) transgene in IL1s. N=3 for each. Error bars represent the SEM. *, **, and *** indicate a significant difference from the wild-type at $p<0.05$, 0.01, and 0.001, respectively, by student *t*-test except for G (a Chi-square test).

Figure 2. *nfya-1* is expressed in and acts in the IL1 neurons to regulate peptidergic and glutamatergic properties. (A) Images of the lateral neurons of adult transgenic animals expressing *nfya-1*p*::gfp* (left) and the *flp-3*p*::mCherry* transgene (middle). Merged images are in the right panel. Anterior is to the left. Scale bar=20μm. (B) Images of the lateral neurons of L2 larval (left) and adult (right) transgenic animals expressing GFP-tagged *nfya-1* cDNA driven under the control of a *nfya-1* promoter that also expresses the *flp-3*p*::mCherry* transgene. Images in the lower left boxed regions (GFP; mCherry; merge) are single-focal-plane confocal microscopy images of the IL1 soma. Anterior is to the left. Scale bar=20μm. (C) Relative levels of *flp-3*p*::gfp* fluorescence in IL1s of wild-type and *ok1174* mutants

expressing *nfya-1* cDNA under the control of *nfya*-1 and *unc-8Δ7* promoters. N=3-5 for each. (D) Percent of animals expressing the *eat-4*p*12::gfp* transgene in IL1s of wild-type and *ok1174* mutants expressing *nfya-1* cDNA under the control of *nfya*-1 and *unc-8Δ7* promoters. n=30–50 for each. (E) *nfya-1* cDNA expression with heat-shock promoter. Heat shocks were treated to egg, L1 or L4 larval stage animals at 33℃ twice for 30 minutes or 35℃ for 2 hours, and after 20 hours, phenotypes were analyzed. N=1-5 for each. (F) Ectopic expression of NFYA-1 using a *flp-7* promoter induces *flp-3* expression in the I5 neuron. Images are derived from z-stacks of confocal microscopy images. Anterior is to the left. Scale bar=20μm. Error bars represent the SEM. *, **, and *** indicate a significant difference from *ok1174* mutant at $p < 0.05$, 0.01, and 0.001, respectively, by a one-way ANOVA with a Tukey post hoc test (C, E, F) and a Chi-square test (D).

Figure 3. Identification of *cis*-regulatory motifs of terminally differentiated IL1 markers. (A-C) The percentage of transgenic animals expressing the *flp-3*p*::gfp* (A) *eat-4*p*::gfp* (B) or *unc-8*p*::gfp* (C) reporter constructs in IL1s is shown. Strength of GFP expression is indicated by the pie charts. Wild-type nucleotides are indicated in black and mutated nucleotides in red. At least two independent extrachromosomal lines for each construct were examined. n≥30 for each. Conserved DNA sequences found in other *Caenorhabditis* species are shown below. (D) ChIP assay with NFYA-1 protein binding to the selected five genes.

Figure 4. The IL1-expressed genes are differentially affected in the IL1 neurons of *nfya-2, nfyb-1,* and *nfyc-1* mutants. (A-B) Representative images (A) and relative expression level of *flp-3*p*::gfp* (B) of wild-type and *nfya-2(tm4194), nfyb-1(cu13),* and *nfyc-1(tm4541)* mutants and *nfya-2;nfya-1* double mutant expressing the *flp-3*p*::gfp* transgene in IL1.

Anterior is to the left. Scale bar=20μm. N=3-5 for each. (C) Relative expression level of *flp-3*p*::gfp* of wild-type and *nfya-1*, *nfya-2*, and *nfya-2;nfya-1* double mutants. N=4-5 for each. (D) Percent of animals expressing the *eat-4*p*12::gfp* transgene in wild-type and *nfya-2*, *nfyb-1*, and *nfyc-1* mutants in IL1. n=40–50 for each. (E) Relative expression level of *unc-8Δ7*p*::gfp* of wild-type and *nfya-2, nfyb-1, nfyc-1,* and *nfya-2;nfya-1* double mutants. N=3 for each. (F) Representative images of animals expressing the *nfyb-1*p*::gfp* transgene. Anterior is to the left. Scale bar=20μm. (G) Model for the functions of the NF-Y subunits in IL1s. Error bars represent the SEM. *, **, and *** indicate a significant difference from wild-type or *ok1174* mutant at $p < 0.05$, 0.01, and 0.001 by a one-way ANOVA with a Tukey post hoc test, respectively, except D (Chi-square test).

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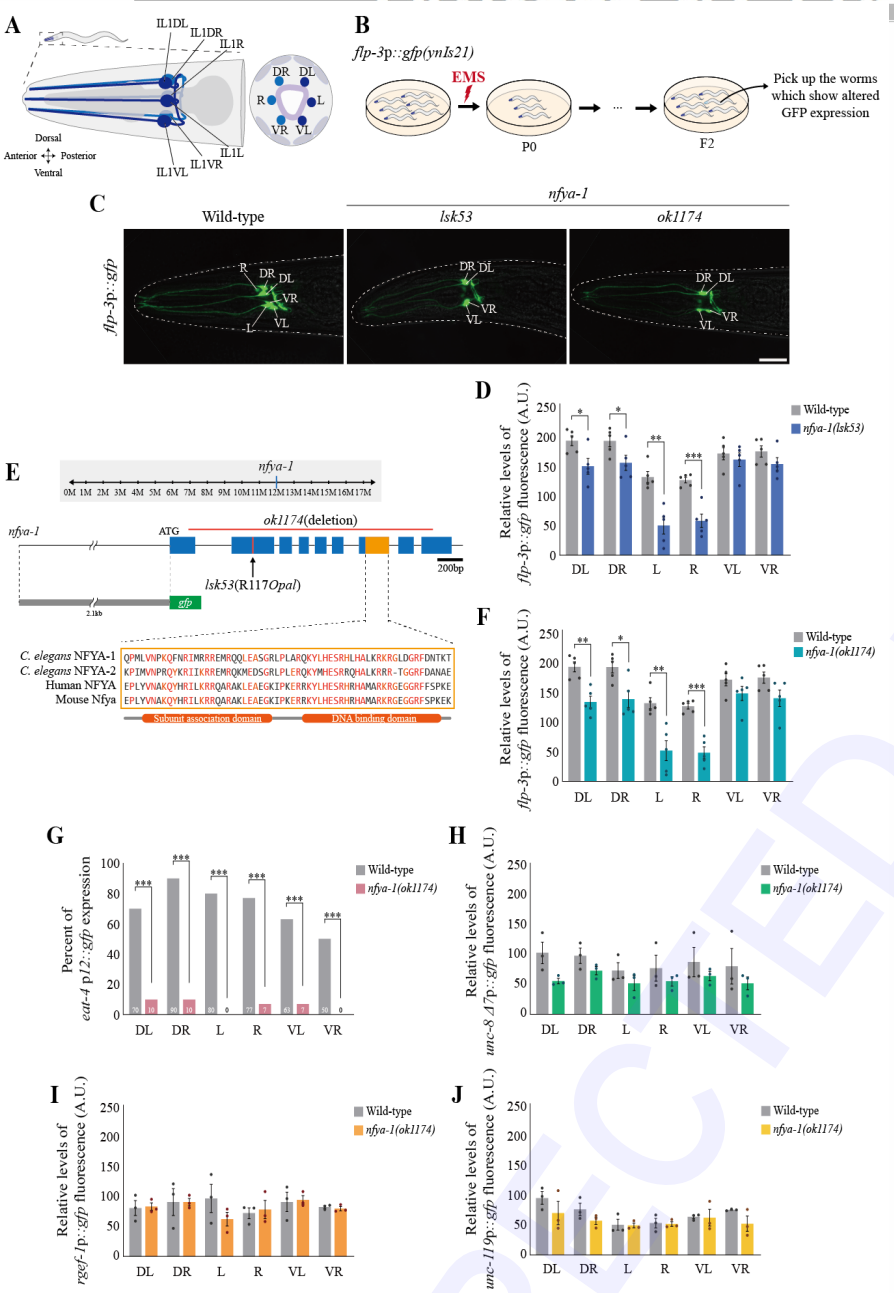

Fig. 1.

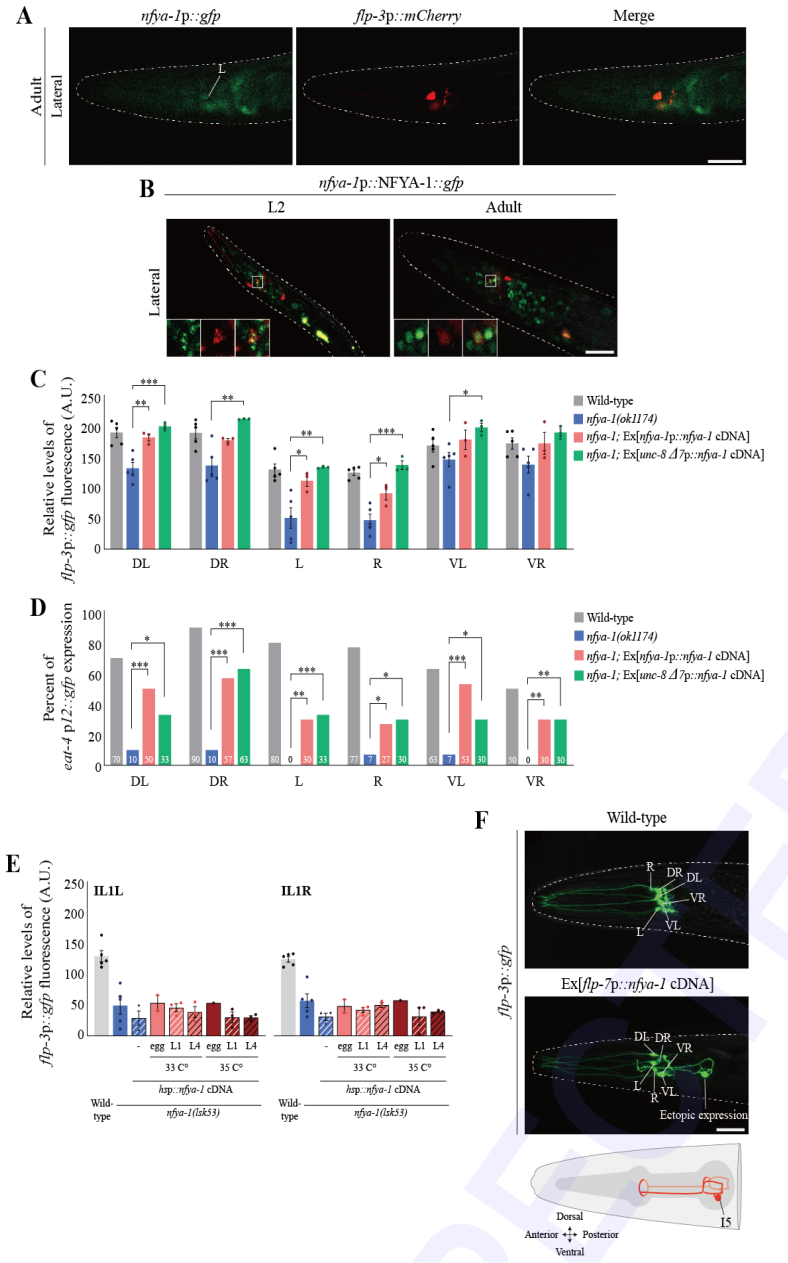

Fig. 2.

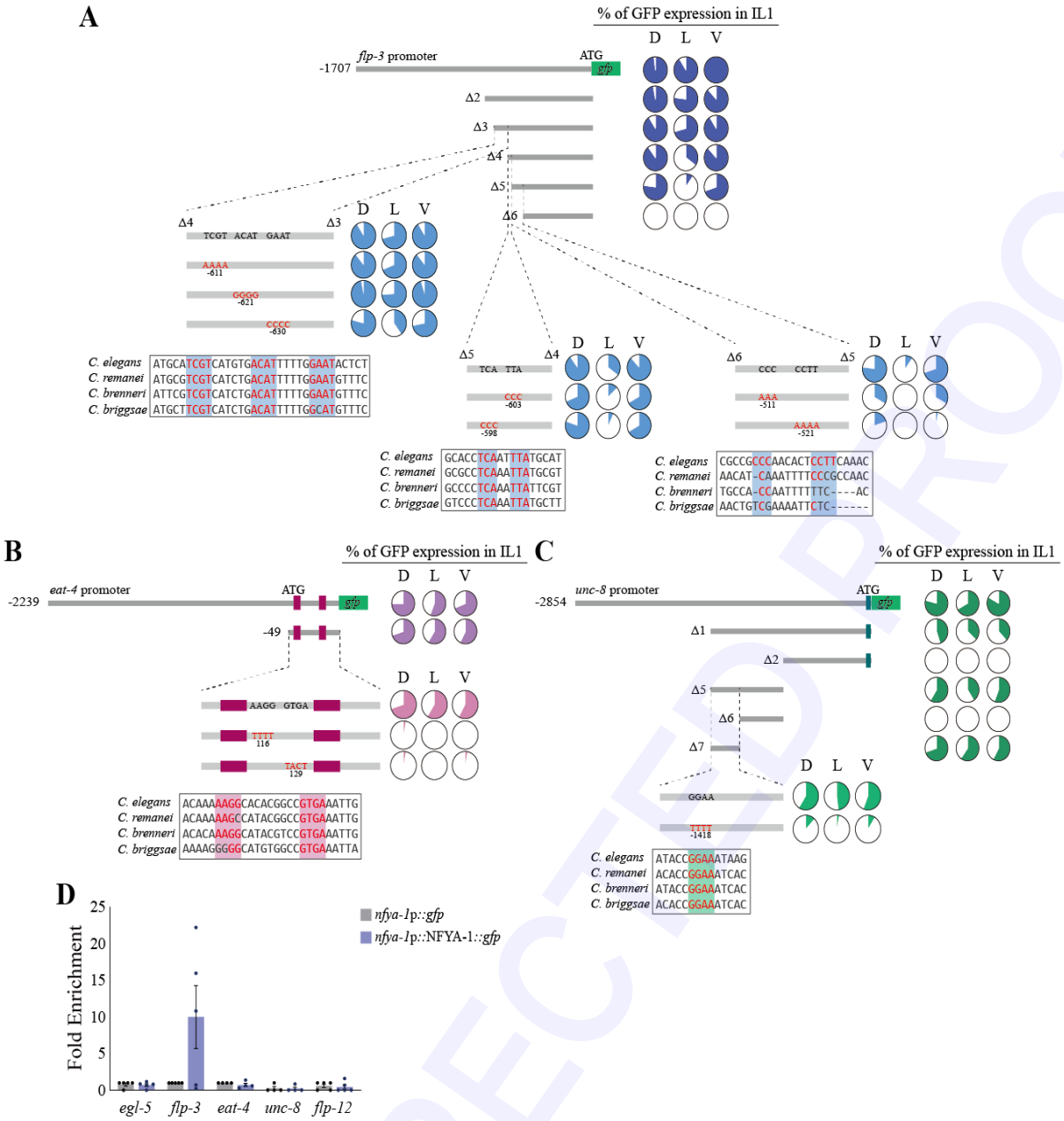

Fig. 3.

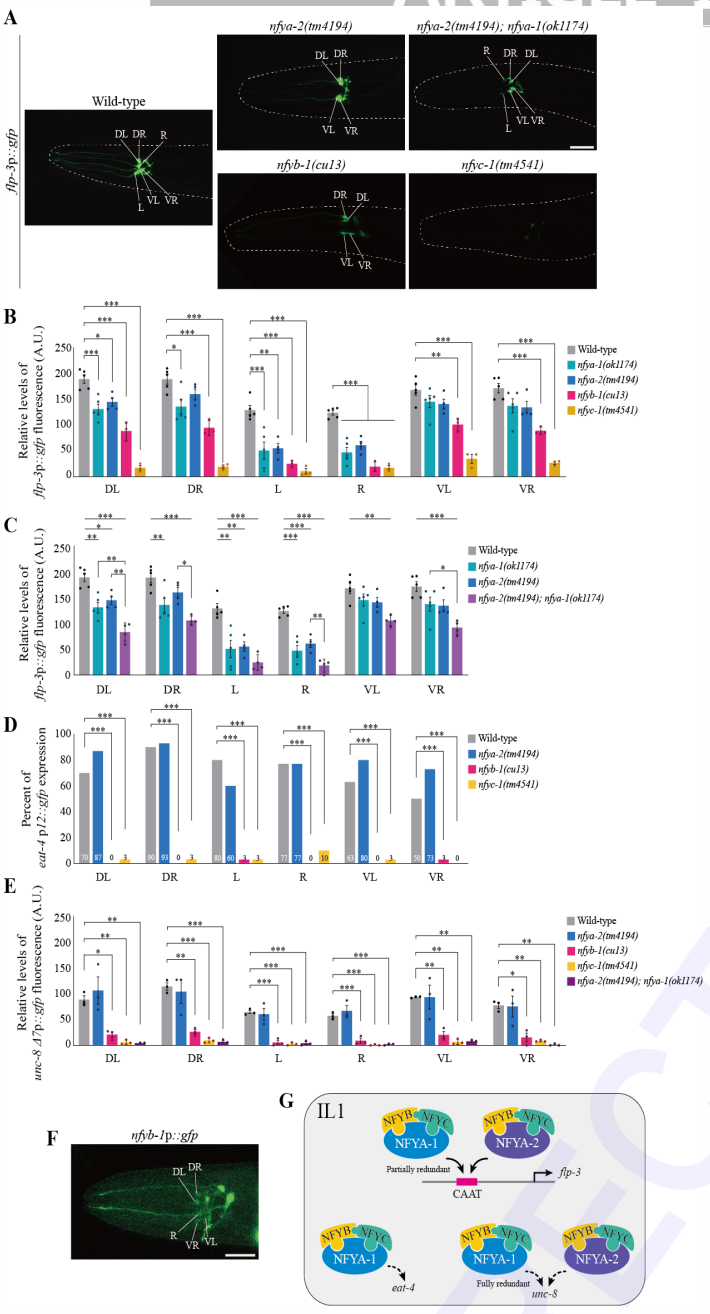

Fig. 4.

DISCUSSION

The heteromeric NF-Y complex consisted of the three subunits, NF-YA, NF-YB, and NF-YC, is an evolutionarily conserved transcription factor in many organisms, including yeast, plants, *Drosophila*, and mammals (31). NF-Y has been shown to regulate various biological processes, including metabolism, cell-cycle progression, and neuronal maintenance (32). In *Drosophila*, it is reported that dNFY, the NF-Y homolog, positively regulates *sevenless*, a receptor tyrosine kinase, and negatively controls a *senseless* transcription factor during photoreceptor differentiation (33). These results indicated that dNFY acts as both an activator and a repressor. In *C. elegans*, it is shown that NF-Y negatively regulates the expression of the transcription factors, *egl-5* hox and *tbx-2* T-box, which are ectopically expressed in *nfy* mutants (18, 19). *egl-5* and *tbx-2* mediate tail morphogenesis and muscle development, respectively (18, 19, 34). These results suggest that *C. elegans* NF-Y acts as a repressor to regulate the expression of transcription factors. In this study, we report that the NF-Y transcription complex is required and responsible for the differentiation and specification of a distinct neuronal class, the IL1 neurons of *C. elegans* (Fig. 4G). The expression of terminally differentiated markers, including the *flp-3* neuropeptide gene, was abolished in *nfy* mutants. In addition, a *cis*-regulated motif found in the *flp-3* gene promoter, which resembles the CCAAT-box identified in other systems (31, 35, 36), appeared to interact with NF-Y directly. These data, together with previous reports, suggest that the *C. elegans* NF-Y also acts as an activator as well as a repressor to mediate developmental processes.

Although *nfya-1* regulates a set of terminally differentiated markers in IL1s, such as *flp-3* neuropeptide and *eat-4* vesicular glutamate transporter, *nfya-1* may not be a usual terminal selector for IL1 specification (8). First, the expression of another terminally differentiated marker, *unc-8* DEG/ENaC cation channel, was not altered in *nfya-1* mutants.

Second, the *cis*-regulatory motifs identified in the *eat-4* and *unc-8* promoters were not similar to the CCAAT-box and directly interacted with NFYA-1 proteins. Third, forced expression of NFYA-1 using the heat-shock promoter in developing larvae and adults did not rescue the phenotype of *nfya-1* mutants. These results, together with previous reports (18, 19), indicate that *nfya-1* may play a role in neuronal differentiation and specification of *C. elegans* not only as a terminal selector but as a proneuronal gene.

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MATERIALS AND METHODS

Strains

All strains were maintained at 20°C. The N2 Bristol was used as a wild-type strain. Mutant strains and transgenic strains used in this study are listed in Supplementary Table. 1.

Isolation of *nfya-1* mutants

The *flp-3*p*::gfp (ynIs21)* integrated strain was used to perform EMS mutagenesis according to Brenner (Brenner, 1974). One mutant allele (*lsk53)* in which GFP expression was abolished in IL1L and IL1R, was isolated and was genetically mapped on LGX. The molecular lesions were further identified by whole-genome sequencing (BGI, see below).

Whole genome resequencing of mutant allele and data analysis

Adult wild-type and *lsk53* mutant animals expressing the *flp-3*p*::gfp(ynIs21)* array were cultured in NGM plates with OP50 at 20°C for 4-5 days. After harvest, worms are lysed and treated with Puregene Tissue Kit (Qiagen) to extract the whole genomic DNA (Doitsidou et al., 2010). Purified genomic DNA of the samples was resequenced at the Beijing Genome Institute. The coverage of whole genome resequencing was 50X and the raw sequencing data was preprocessed and aligned onto *C. elegance* reference genome (WBcel235/ce11 assembly) using Burrows-Wheeler Aligner (Li and Durbin, 2009). Using the post-processed alignment results, SNP and indels were called with the Genome Analysis Toolkit (GATK) (Van der Auwera et al., 2013). To annotate the genomic variants, SnpEff with Ensembl release 86 was used and the results were filtered based on ‘stop-gained’ annotation (premature translation

stop). In total 9 'stop-gained' SNPs in 8 genes (Supplementary Table. 2) were identified in *lsk53* mutants.

Molecular biology and transgenic worms

All the constructs created in this study were inserted into the pPD95.77 vector and its derivatives. For the *flp-3, eat-4,* and *unc-8* promoter analysis, 1.7 kb, 500 bp, and 2.8 kb promoter regions were amplified by PCR from N2 genomic DNA, respectively (Kim and Li, 2004; Serrano-Saiz et al., 2013). For a promoter analysis, each promoter region was deleted with various digestion enzymes or PCR fusions. To generate transgenic worms, 50 ng of each reporter construct was injected, with 50 ng of *unc-122*p*::dsRed* as an injection marker. Mutagenesis was performed using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. For the constructs used for rescue experiment, the *nfya-1, rgef-1, unc-8 Δ7,* and *hsp16.2* promoters were fused with *nfya-1* cDNA isolated from N2 cDNA library. With 50 ng of *unc-122*p*::gfp* as an injection marker, 2 or 50 ng of *nfya-1* cDNA under the control of various promoters were injected into *nfya-1* mutant alleles. For ectopic expression, *flp-7* promoter was fused with *nfya-1* cDNA and injected into *flp-3*p*::gfp (ynIs21)* with 50 ng of *unc-122*p*::gfp* as an injection marker.

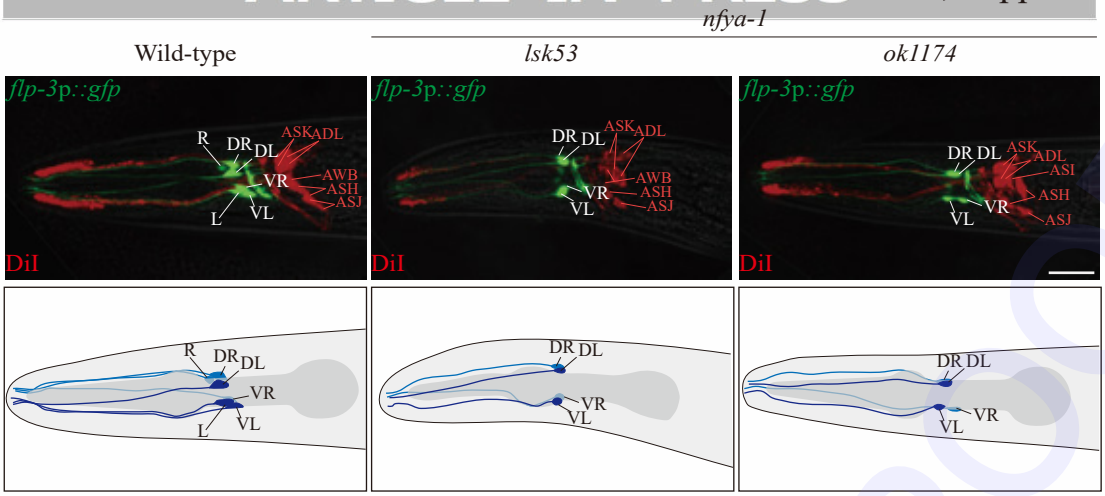
Quantification of GFP expression

For GFP quantification, the worms were anesthetized in sodium azide on an agar pad, and GFP fluorescence was observed with Zeiss Axio Imager using 40X. Animals that have *rol-6* marker were dye-filled with DiI to identify the dorsoventral and left-right axis before the test. For promoter analysis, the relative GFP levels were rated from 0 (no expression) to 5 (bright)

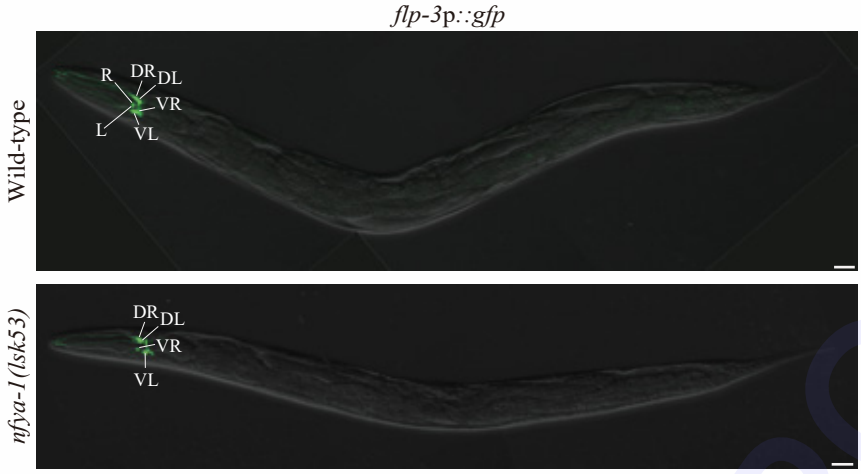
in every six IL1 cells by visual inspection. The relative expression level of GFP was measured and these values were analyzed using Image J software.

ChIP (Chromatin immunoprecipitation) assay

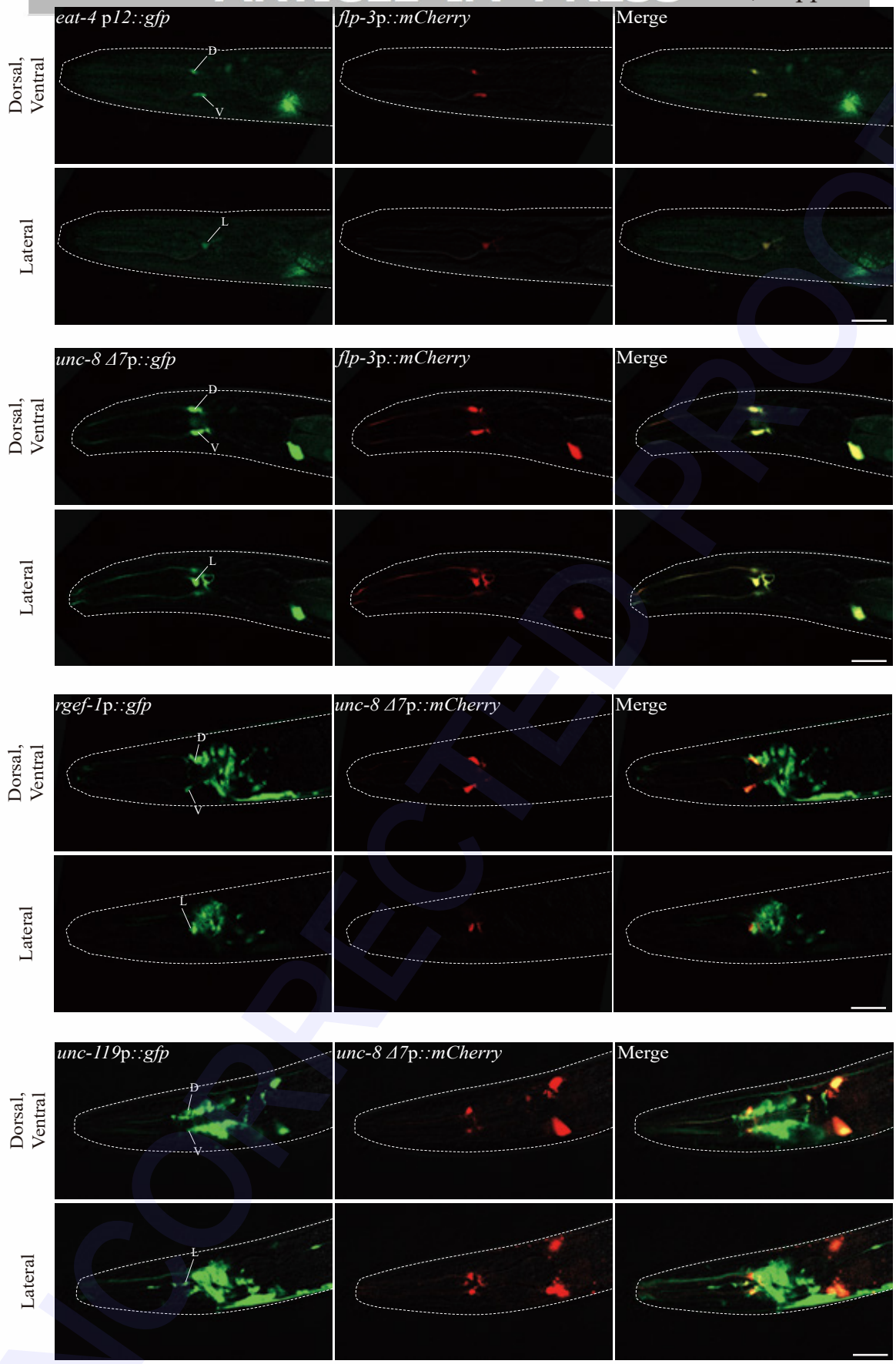
Adult worms expressing *nfya-1*p*::gfp* or *nfya-1*p*::*NFYA-1*::gfp* array, were cultured in NGM plates with OP50 at 20°C for 4-5 days. After harvest, worms are homogenized and incubated with 1.1 % of formaldehyde/PBS buffer to cross-link proteins to DNAs. The chromatin of worms was sheared to shorter DNA fragments by sonication, and protein-DNA fragments were immunoprecipitated using GFP-Trap® Magnetic Particles M-270 (Chromotek). After reverse cross-linking, the samples were treated with proteinase K and DNA fragments were precipitated out from the extracted solution. Quantitative PCR was performed using the SYBR Green PCR master mix kit (Applied Biosystems).



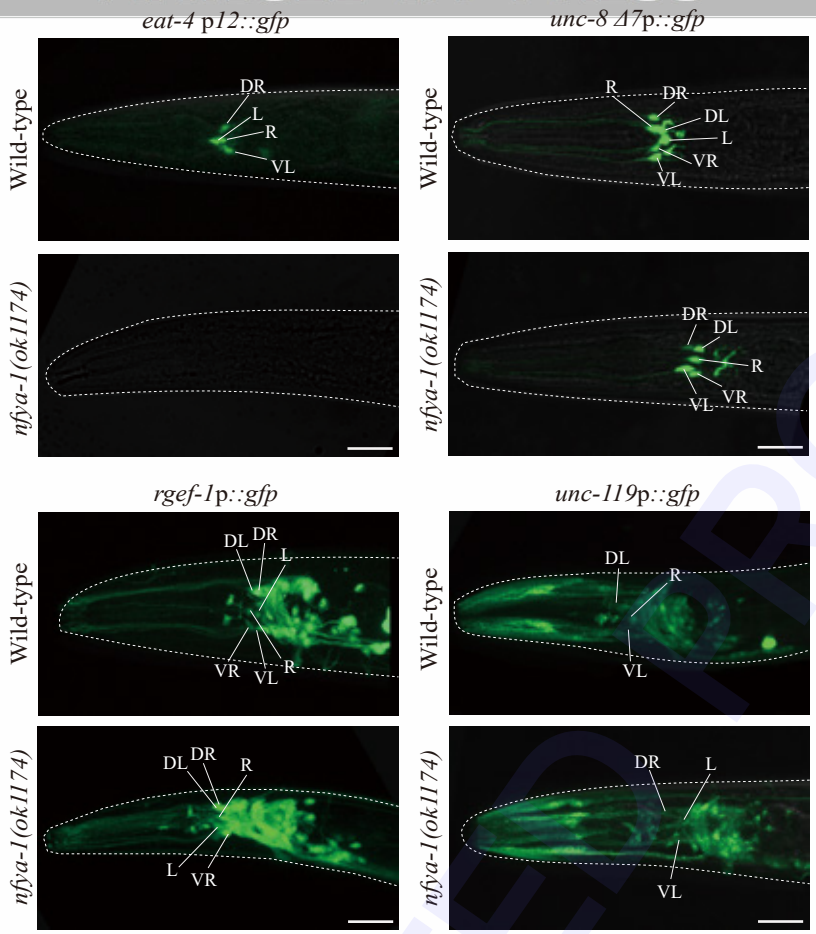
Supplementary Fig. 1. Images of DiI dye-filled wild-type and *nfya-1* mutant (*lsk53* and *ok1174*) animals expressing the *flp-3*p*::gfp* transgene in the IL1 neurons (Top). Anterior is at the left in all images. Scale bar = 20 μm. Schematic drawing of the IL1 neurons in *C. elegans* in wild-type and *nfya-1* mutant alleles (bottom).



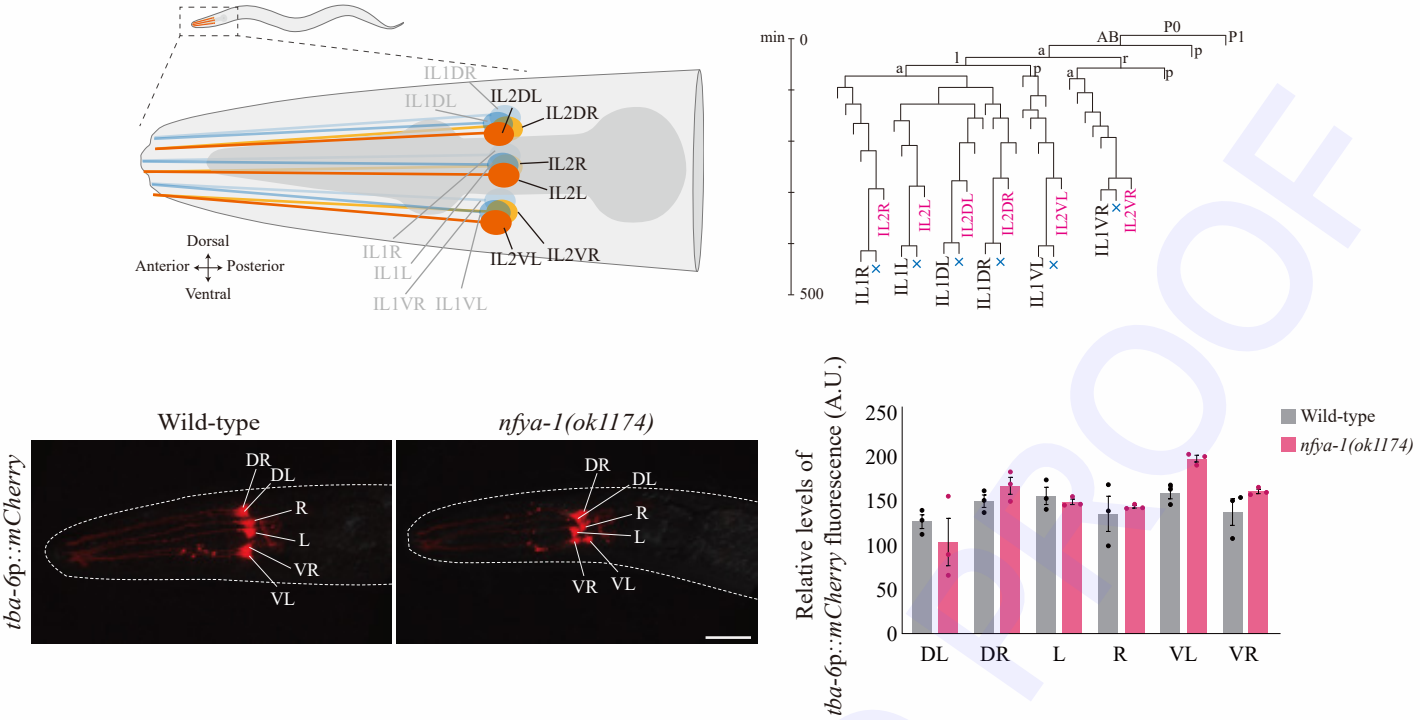
Supplementary Fig. 2. Adult whole-body images of wild-type and *nfya-1*(*lsk53*) mutant animals expressing the *flp-3*p*::gfp* transgene. Scale bar = 20 μm.



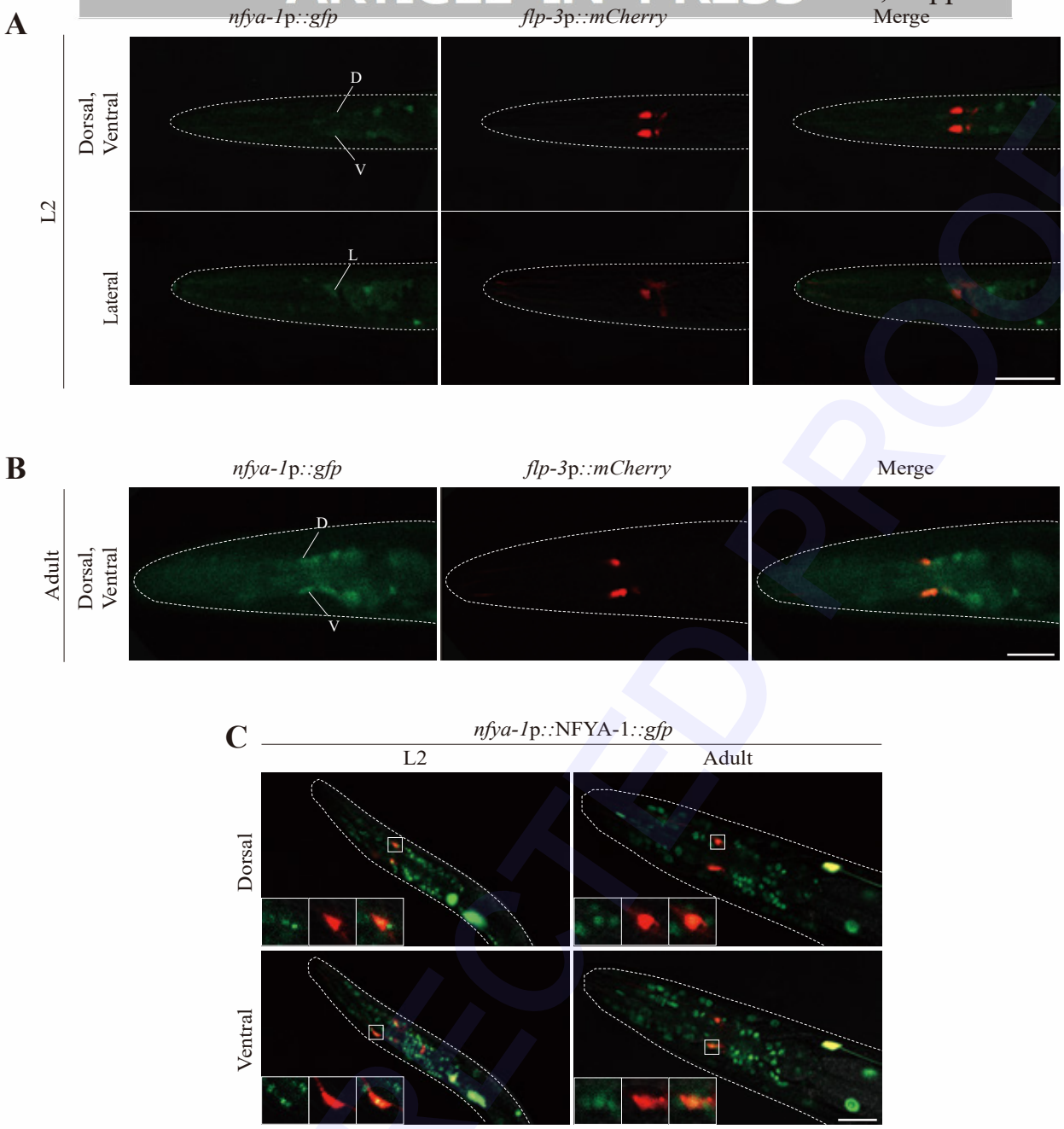
Supplementary Fig. 3. Images of transgenic animals expressing the *eat-4* p*12::gfp, unc-8 Δ7*p*::gfp, rgef-1*p*::gfp,* or *unc-119*p*::gfp* transgenes (left) that also express the *flp-3*p*::mCherry* or *unc-8 Δ7*p*::mCherry* transgenes (middle). Merged images are in the right panel. Images were taken in two different focal planes to visualize the dorsal, ventral, and lateral IL1s. Scale bar = 20 μm.



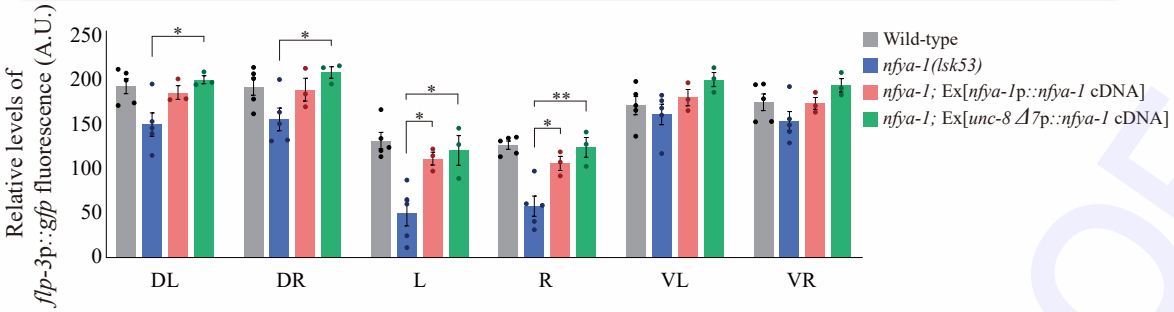
Supplementary Fig. 4. Representative images of wild-type and *nfya-1*(*ok1174*) mutant animals expressing the *eat-4* p*12::gfp, unc-8 Δ7*p*::gfp, rgef-1*p*::gfp,* or *unc-119*p*::gfp* transgene in the IL1 neurons. Anterior is th the legt. Scale bar = 20 μm.



Supplementary Fig. 5. Schematic drawing (upper left) and cell lineage (upper right) of the IL1 and IL2 neurons in *C. elegans*. Representative images of wild-type and *nfya-1(ok1174)* mutant animals expressing the *tba-6*p*::mCherry* transgene (bottom left). The relative level of *tba-6*p*::mCherry* transgene in wild-type and *nfya-1(ok1174)* mutant animals (bottom right). N = 3 for each (ten worms per N). Error bars represent the SEM. *, **, and *** indicate a significant difference from wild-type at $p < 0.05$, 0.01, and 0.001 by a student t-test, respectively.

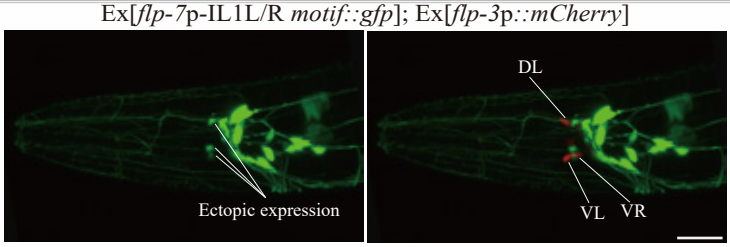


Supplementary Fig. 6. (A) Images of L2 larval transgenic animals expressing *nfya-1*p*::gfp* (left) that also express the *flp-3*p*::mCherry* transgene (middle). Merged images are in the right panel. Images were taken in two different focal planes to visualize the dorsal, ventral, and lateral IL1s. (B) Images of adult transgenic animals expressing *nfya-1*p*::gfp* (left) that also express the *flp-3*p*::mCherry* transgene (middle) in dorsal and ventral IL1s. (C) Images of dorsal and ventral IL1s of L2 larval (left) and adult (right) transgenic animals expressing GFP-tagged *nfya-1* cDNA driven under the control of a *nfya-1* promoter that also expresses the *flp-3*p*::mCherry* transgene. Images in the lower left boxed regions (GFP; mCherry; merge) are single-focal-plane confocal microscopy images of the IL1 soma. Anterior is to the left. Scale bar = 20 μm.

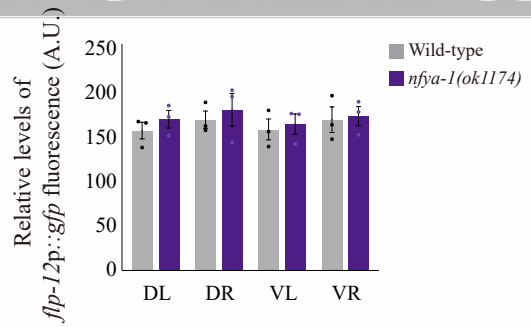


Supplementary Fig. 7. Relative levels of *flp-3*p*::gfp* fluorescence in IL1s of wild-type and *lsk53* mutant animals expressing *nfya-1* cDNA under the control of the *nfya-1* and *unc-8 Δ7* (IL1 cell-specific promoter) promoters. N = 3-5 for each (ten worms per N). Error bars represent the SEM. *, **, and *** indicate a significant difference from *lsk53* mutant at $p < 0.05$, 0.01, and 0.001 by a one-way ANOVA with a Tukey post hoc test, respectively.

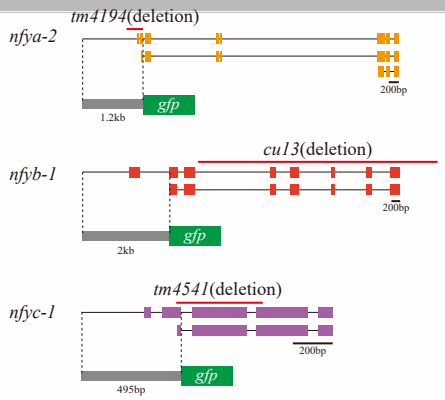
Ex[*flp-7*p-IL1L/R *motif::gfp*]; Ex[*flp-3*p*::mCherry*]



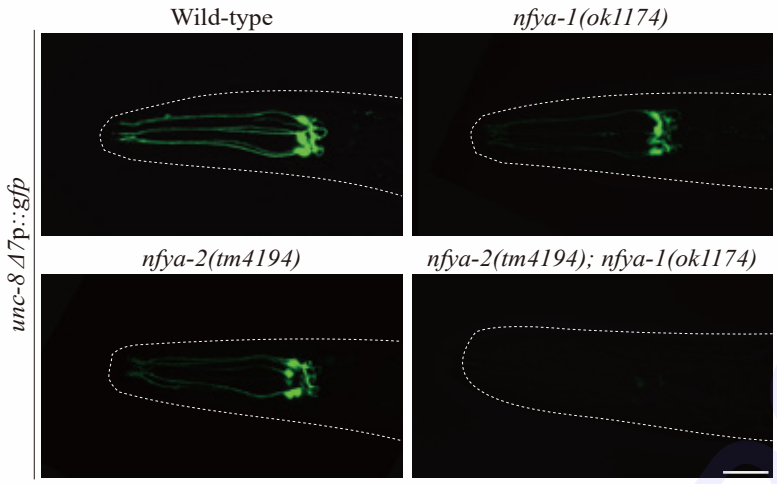
Supplementary Fig. 8. Insertion of the IL1L/R motif into the non-IL1 expressed *flp-7* gene promoter induces *flp-7* expression in other neuron-types (40). Images are derived from z-stacks of confocal microscopy images. Anterior is to the left. Scale bar = 20 μm.



Supplementary Fig. 9. Relative levels of the *flp-12*p*::gfp* reporter of wild-type*(ynIs25)* and *nfya-1(ok1174)* mutants in the SMB neurons. N = 3 for each. Error bars represent the SEM. *, **, and *** indicate a significant difference from wild-type at $p < 0.05$, 0.01, and 0.001 by a student t-test, respectively.



Supplementary Fig. 10. Genomic structure of *nfya-2* (upper), *nfyb-1* (middle), and *nfyc-1* (bottom).



Supplementary Fig. 11. Representative images of wild-type and *nfya-1(ok1174), nfya-2(tm4194),* and *nfya-2;nfya-1* double mutant animals expressing the *unc-8 Δ7*p*::gfp* transgene in the IL1 neurons. Anterior is to the left. Scale bar = 20 μm.

Gene	Genotype	Name
Ex[*eat-4* p*12::gfp pm3;unc-122* p::*dsRed*]	*lskEx1090*	KHK1585
Ex[*eat-4* p*12::gfp pm4;unc-122* p::*dsRed*]	*lskEx1092*	KHK1587
Ex[*eat-4* p*12::gfp;unc-122* p::*dsRed*]	*lskEX1017*	KHK1457
Ex[*eat-4* p*12::gfp*];Ex[*flp-3* p::*mCherry*]		KHK2249
Ex[*eat-4* p::*gfp;unc-122* p::*dsRed*]	*lskEx1584*	KHK2209
Ex[*flp-3* Δ2p::*gfp;unc-122* p::*dsRed*]	*lsk Ex190*	KHK197
Ex[*flp-3* Δ3p::*gfp pm21;unc-122* p::*dsRed*]	*lskEx926*	KHK1325
Ex[*flp-3* Δ3p::*gfp pm22;unc-122* p::*dsRed*]	*lskEx927*	KHK1326
Ex[*flp-3* Δ3p::*gfp pm23;unc-122* p::*dsRed*]	*lskEx916*	KHK1313
Ex[*flp-3* Δ3p::*gfp;unc-122* p::*dsRed*]	*lskEx908*	KHK1300
Ex[*flp-3* Δ4p::*gfp pm3;unc-122* p::*dsRed*]	*lskEx479*	KHK707
Ex[*flp-3* Δ4p::*gfp pm7;unc-122* p::*dsRed*]	*lskEx458*	KHK683
Ex[*flp-3* Δ4p::*gfp;unc-122* p::*dsRed*]	*lskEx888*	KHK1272
Ex[*flp-3* Δ5p::*gfp pm15;unc-122* p::*dsRed*]	*lskEx1368*	KHK1931
Ex[*flp-3* Δ5p::*gfp pm27;unc-122* p::*dsRed*]	*lskEx1381*	KHK1948
Ex[*flp-3* Δ5p::*gfp;unc-122* p::*dsRed*]	*lskEx897*	KHK1283
Ex[*flp-3* Δ6p::*gfp;unc-122* p::*dsRed*]	*lskEx913*	KHK1305
Ex[*flp-3* p::*gfp;unc-122* p:*dsRed*]	*lsk Ex354*	KHK367
Ex[flp-3p::mCherry;unc-122p::gfp]	*lskEx497*	KHK725
Ex[*flp-7* p-IL1L/R motif 3X insert::*gfp;unc-122* p::*dsRed*]	*lskEX1015*	KHK1455
Ex[*nfya-1* p::*gfp;unc-122* p::*dsRed*]	*lskEx1504*	KHK2102
Ex[*nfya-1* p::*gfp;unc-122* p::*dsRed*];Ex[*flp-3* p::*mcherry;unc-122* p::*gfp*]		KHK2166
Ex[*nfya-1* p::*gfp;unc-122* p::*dsRed*];Ex[*unc-8* Δ7p::*mCherry;unc-122* p::*gfp*]		KHK2205
Ex[*nfya-1* p::*nfya-1* cDNA::*gfp;unc-122* p::*dsRed*]	*lskEx1548*	KHK2162
Ex[*nfyb-1* p::*gfp;unc-122* p::*dsRed*]	*lskEx1642*	KHK2307
Ex[*nfyb-1* p::*gfp;unc-122* p::*dsRed*];Ex[*flp-3* p::*mCherry;unc-122* p::*gfp*]		KHK2305
Ex[*rgef-1* p::*gfp*]	*lskEx1366*	KHK1924
Ex[*rgef-1* p::*gfp*];Ex[*unc-8* Δ7p::*mCherry*]		KHK2247
Ex[*tba-6* p::*mCherry;unc-122* p::*gfp*]	*lskEX1104*	KHK1600
Ex[*unc-8* Δ1p::*gfp;unc-122* p::*dsRed*]	*lskEX1023*	KHK1476
Ex[unc-8 Δ2p::gfp;unc-122p::dsRed]	*lskEx1191*	KHK1726
Ex[*unc-8* Δ5p::*gfp pm6;unc-122* p::*dsRed*]	*lskEx1374*	KHK1937
Ex[*unc-8* Δ5p::*gfp;unc-122* p::*dsRed*]	*lskEx1210*	KHK1752
Ex[*unc-8* Δ6p::*gfp;unc-122* p::*dsRed*]	*lskEX1344*	KHK1899
Ex[*unc-8* Δ7p::*gfp;unc-122* p::*dsRed*]	*lskEx1375*	KHK1938
Ex[*unc-8* Δ7p::*gfp*];Ex[*flp-3* p::*mcherr* y]		KHK2248
Ex[*unc-8* Δ7p::*mCherry;unc-122* p::*gfp*]	*lskEx1634*	KHK2218
Ex[*unc-8* p::*gfp;unc-122* p::*dsRed*];Ex[*flp-3* p::*mcherry;unc-122* p::*gfp*]		KHK1140
Is [*flp-12* p::*gfp*]	*ynIs25*	NY2025
Is [*flp-3* p::*gfp;rol-6*]	*ynIs21*	
Is [*flp-3* p::*gfp*];Ex[*flp-7* p::*nfya-1* cDNA;*unc-122* p::*gfp*]	*lskEx1473*	KHK2057
nfya-1	*lsk53*	KHK2113
nfya-1	*ok1174*	VC763
nfya-1 (*lsk53*);Ex[*hs* p::*nfya-1* cDNA;*unc-122* p::*gfp*]	*lskEx1503*	KHK2097
nfya-1 (*lsk53*);Ex[*nfya-1* p::*nfya-1* cDNA;*unc-122* p::*gfp*]	*lskEx1490*	KHK2081
nfya-1 (*lsk53*);Ex[*unc-8* Δ7p::*nfya-1* cDNA;*unc-122* p::*gfp*]	*lskEx1586*	KHK2211
nfya-1 (*ok1174*);Ex[*eat-4* p*12::gfp;unc-122* p::*dsRed*]		KHK2047
nfya-1 (*ok1174*);Ex[*eat-4* p*12::gfp;unc-122* p::*dsRed*];Ex[*unc-8* Δ7p::*nfya-1* cDNA]		KHK2239
nfya-1 (*ok1174*);Ex[*eat-4* p::*gfp;unc-122* p::*dsRed*];Ex[*nfya-1* p::*nfya-1* cDNA;*unc-122* p::*gfp*]	*lskEx1603*	KHK2240
nfya-1 (*ok1174*);Ex[*nfya-1* p::*gfp;unc-122* p::*dsRed*]		KHK2167

nfya-1 (*ok1174*);Ex[*nfya-1* p*::gfp;unc-122* p*::dsRed*];Ex[*unc-8* Δ7p*::mCherry;unc-122* p*::gfp*]		KHK2206
nfya-1 (*ok1174*);Ex[*nfya-1* p*::nfya-1* cDNA*;unc-122* p*::gfp*]	*lskEx1659*	KHK2333
nfya-1 (*ok1174*);Ex[*rgef-1* p*::gfp*];Ex[*unc-8* Δ7p*::mCherry*]		KHK2330
nfya-1 (*ok1174*);Ex[*tba-6* p*::mCherry;unc-122* p*::gfp*]		KHK2214
nfya-1 (*ok1174*);Ex[*unc-8* Δ7p*::gfp;unc-122* p*::dsRed*]		KHK2079
nfya-1 (*ok1174*);Ex[*unc-8* Δ7p*::nfya-1* cDNA*;unc-122* p*::gfp*]	*lskEx1576*	KHK2196
nfya-1 (*ok1174*);*Is* [*flp-12* p*::gfp*]		KHK2271
nfya-1 (*ok1174*);*Is* [*flp-3* p*::gfp;rol-6*]		KHK2048
nfya-1 (*ok1174*);*Is* [*flp-3* p*::gfp*];Ex[*unc-8* Δ7p*::nfya-1* cDNA;*unc-122* p*::gfp*]	*lskEx1631*	KHK2215
nfya-1 (*ok1174*);*unc-119* p*::gfp* ;Ex[*unc-8* Δ7p*::mCherry*]		KHK2329
nfya-2	*tm4194*	
nfya-2 (*tm4194*);Ex[*eat-4* p*12::gfp;unc-122* p*::dsRed*]		KHK2313
nfya-2 (*tm4194*);Ex[*unc-8* Δ7p*::gfp;unc-122* p*::dsRed*]		KHK2304
nfya-2 (*tm4194*);*Is* [*flp-3* p*::gfp*]		KHK2253
nfya-2 (*tm4194*);*nfya-1* (*ok1174*);Ex[*unc-8* Δ7p*::gfp;unc-122* p*::dsRed*]		KHK2327
nfya-2 (*tm4194*);*nfya-1* (*ok1174*);*Is* [*flp-3* p*::gfp*]		KHK2300
nfyb-1	*cu13*	OK814
nfyb-1 (*cu13*);Ex[*eat-4* p*12::gfp;unc-122* p*::dsRed*]		KHK2309
nfyb-1 (*cu13*);Ex[*unc-8* Δ7p*::gfp;unc-122* p*::dsRed*]		KHK2303
nfyb-1 (*cu13*);*Is* [*flp-3* p*::gfp*]		KHK2301
nfyc-1	*tm4541*	
nfyc-1 (*tm4541*);Ex[*eat-4* p*12::gfp;unc-122* p*::dsRed*]		KHK2257
nfyc-1 (*tm4541*);Ex[*unc-8* Δ7p*::gfp;unc-122* p*::dsRed*]		KHK2266
nfyc-1 (*tm4541*);*Is* [*flp-3* p*::gfp*]		KHK2265
unc-119 p*::gfp*	*otls45*	OH441
unc-119 p*::gfp* ;Ex[*unc-8* Δ7p*::mCherry*]		KHK2246

Chromosome	Position	REF	ALT	Feature_ID	Feature Type	Annotation	Annotation Impact	HGVS.p	FORMAT	*lsk53*	GO term description
chrI	4765802	G	T	M04F3.2	transcript	stop_gained	HIGH	p.Glu17*	GT:AD:DP:GQ:PGT:PID:PL	0/1:14,26:40:99:1|0:4765756_G_A:1030,0,510	ribosomal large subunit assembly, rRNA binding, preribosome, large subunit precursor
chrI	5162917	A	T	let-630	transcript	stop_gained	HIGH	p.Tyr588*	GT:AD:DP:GQ:PL	0/1:110,28:138:99:597,0,4077	
chrII	5731707	C	A	C18A3.11	transcript	stop_gained	HIGH	p.Ser212*	GT:AD:DP:GQ:PL	0/1:43,8:53:85:85,0,1274	
chrIV	16787420	A	T	Y43D4A.6	transcript	stop_gained	HIGH	p.Lys32*	GT:AD:DP:GQ:PL	0/1:105,115:220:99:4044,0,3392	nucleotide binding, protein kinase activity, protein serine/threonine kinase activity, ATP binding, protein phosphorylation, kinase activity, phosphorylation, transferase activity
chrV	6674509	G	A	K11G9.2	transcript	stop_gained	HIGH	p.Trp63*	GT:AD:DP:GQ:PL	1/1:0,148:148:99:5797,445,0	metabolic process, hydrolase activity, carboxylic ester hydrolase activity, extracellular space
chrX	5258139	T	A	Y34B4A.5	transcript	stop_gained	HIGH	p.Tyr176*	GT:AD:DP:GQ:PL	1/1:0,98:98:99:3833,294,0	
chrX	8846738	G	A	C28G1.t2	transcript	stop_gained	HIGH	p.Trp6*	GT:AD:DP:GQ:PL	0/1:111,45:156:99:1326,0,4152	None
chrX	8846775	C	T	C28G1.t2	transcript	stop_gained	HIGH	p.Arg19*	GT:AD:DP:GQ:PGT:PID:PL	0/1:105,34:139:99:0|1:8846775_C_T:1090,0,4235	None
chrX	11946168	G	A	nfya-1	transcript	stop_gained	HIGH	p.Arg117*	GT:AD:DP:GQ:PL	1/1:2,99:101:99:3793,211,0	nucleus, transcription factor activity, sequence-specific DNA binding, regulation of transcription, DNA-templated