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The NF-Y Complex negatively regulates *C. elegans tbx-2* expression

Angenee C. Milton[1], **Adelaide V. Packard**[1], **Lynn Clary**[1,2], and **Peter G. Okkema**[1,3]
[1]Laboratory for Molecular Biology, Department of Biological Sciences, University of Illinois at Chicago, 900 S. Ashland Avenue (MC567), Chicago, IL 60607, USA

Abstract

T-box genes are frequently expressed in dynamic patterns during animal development, but the mechanisms controlling expression of these genes are not well understood. The *C. elegans* T-box gene *tbx-2* is essential for development of the ABa-derived pharyngeal muscles, specification of neural cell fate in the HSN/PHB lineage, and adaptation in olfactory neurons. The *tbx-2* expression pattern is complex, and expression has been described in pharyngeal precursors and body wall muscles during embryogenesis, and amphid sensory neurons and pharyngeal neurons in adults. To examine mechanisms regulating *tbx-2* gene expression, we performed an RNAi screen of transcription factor genes in strains containing a *Ptbx-2::gfp* reporter and identified the Nuclear Factor Y (NF-Y) complex as a negative regulator of *tbx-2* expression. NF-Y is a heterotrimeric CCAAT-binding complex consisting of A, B and C subunits, and reduction of the NF-Y subunits *nfya-1*, *nfyb-1*, or *nfyc-1* by RNAi or using mutants results in ectopic *Ptbx-2::gfp* expression in hypodermal seam cells and gut. Mutation of two CCAAT-boxes in the *tbx-2* promoter results in a similar pattern of ectopic *Ptbx-2::gfp* expression, suggesting NF-Y directly represses the *tbx-2* promoter. *tbx-2* mRNA is moderately increased in *nfya-1* null mutants, indicating NF-Y represses expression of endogenous *tbx-2*. Finally we identify and characterize a second-site mutation that enhances lethality of a temperature sensitive *tbx-2* mutant and show that this mutation is a deletion in the *nfyb-1* gene. Together, these results identify NF-Y as an important regulator of *tbx-2* function *in vivo.*

Keywords

C. elegans; NF-Y; T-box gene; transcriptional repression; *tbx-2*

Introduction

T-box transcription factors play essential roles in the development of all multicellular animals, where their functions include the specification of primary germ layers and the specification of cell fates during organogenesis [reviewed in (Naiche et al., 2005; Showell et al., 2004)]. In humans, mutations in T-box genes have recently been implicated in a variety of congenital diseases and cancers, and, in most cases, the level of T-box gene expression is

[3]Corresponding author: Peter Okkema, Laboratory for Molecular Biology, Department of Biological Sciences, University of Illinois at Chicago, 900 S. Ashland Avenue (MC567), Chicago, IL 60607, USA, okkema@uic.edu, tel. 1-312-413-7445, fax 1-312-413-2691.
[2]Current address: Department of Biological Sciences, Harold Washington College, Chicago, IL USA

a crucial determinant of disease. For example, haploinsufficiency for the T-box genes *TBX5, TBX3, TBX1, TBX22*, and *TBX19* results in Holt-Oram syndrome, Ulnar-Mammary syndrome, DiGeorge syndrome, cleft palate with ankyloglossia, and ACTH deficiency, respectively [reviewed in (Packham and Brook, 2003)]. Conversely, overexpression of *TBX2* and *TBX3* is observed in a variety of cancers, including cancers of the breast, pancreas, ovary, and skin [reviewed in (Abrahams et al., 2010; Lu et al., 2010)]. Because precise regulation of T-box gene expression is crucial for normal function, several recent studies have focused on identifying upstream regulators of mammalian T-box genes. These have identified Wnt/β-catenin and BMP/Smad signaling pathways, the cell cycle regulators PML and E2F4, and the Nuclear Factor Y (NF-Y) complex as regulators of T-box gene expression (Martin et al., 2012; Renard et al., 2007; Smith et al., 2011; Teng et al., 2008; Zhang et al., 2011).

We are characterizing mechanisms controlling T-box factor expression and activity in *C. elegans* and are focusing on the *tbx-2* gene. TBX-2 is the sole *C. elegans* member of the Tbx2-subfamily of T-box factors, and it is most closely related to the human proteins TBX2 and TBX3 (Pocock et al., 2004; Roy Chowdhuri et al., 2006). During embryogenesis *tbx-2* is required for formation of pharyngeal muscles derived from the ABa blastomere and for specifying the fate and differentiation pattern of the HSN and PHB neurons, and null mutants exhibit larval lethality resulting from an inability to feed (Roy Chowdhuri et al., 2006; Singhvi et al., 2008; Smith and Mango, 2007). *tbx-2* also plays a role in neuronal plasticity, and viable non-null alleles have defects in adaptation to odorants and formation of dauer larvae (Miyahara et al., 2004). *tbx-2* null mutants arrest as L1 larvae shortly after hatching due to pharyngeal defects and an inability to feed (Roy Chowdhuri et al., 2006; Smith and Mango, 2007)

Consistent with its diverse spatial and temporal functions, *tbx-2* is expressed in a variety of tissues. *tbx*-2 expression has been examined at different developmental stages using *gfp* reporters (Roy Chowdhuri et al., 2006; Smith and Mango, 2007), antibodies (Miyahara et al., 2004), and by *in situ* hybridization [NEXTDB Ver. 4.0, yk112c4, http://nematode.lab.nig.ac.jp/db/index.html; (Kohara, 2001)]. These analyses indicate *tbx-2* is expressed in a dynamic pattern, with strong expression in the developing pharynx during embryogenesis and expression in a subset of neurons from late embryogenesis through adulthood. *tbx-2* expression outside the pharynx is repressed by LAG-1 and REF-1 components of the Notch signaling pathway, while expression in the pharynx is maintained by PHA-4/FoxA in a positive feedback loop (Smith and Mango, 2007).

To identify additional mechanisms controlling *tbx-2* expression, we performed an RNAi screen of transcription factor genes in which we looked for altered expression of a *tbx-2::gfp* reporter. We found that RNAi knockdown of the *nfyc-1* subunit of the CCAAT-box binding factor NF-Y resulted in ectopic *tbx-2::gfp* expression in the seam cells and gut. *nfyc-1* encodes one subunit of the heterotrimeric NF-Y transcription factor. We used a variety of genetic and molecular approaches that indicate the NF-Y complex containing the NFYA-1, NFYB-1 and NFYC-1 subunits directly targets the *tbx-2* promoter and represses *tbx-2* expression. Further, we found strong genetic interactions between a newly identified *nfyb-1* mutant and a temperature sensitive *tbx-2* mutant. Together, these results indicate NF-Y repression is crucial for *tbx-2* function *in vivo*.

Materials and Methods

Nematode handling, transformation, and strains

C. elegans were grown under standard conditions (Lewis and Fleming, 1995). Germ line transformation was performed by microinjection with pRF4 containing *rol-6(su1006)* as a

transformation marker (100 ng/μl) with *gfp* reporters (10 ng/μl) (Mello and Fire, 1995). The following strains were used in these studies: OK0592 *cuIs23[Ptbx-2::gfp] III*, OK0969 *rrf-3(pk1426) II; cuIs23 III* (Simmer et al., 2002), OK0029 *cuIs1[ceh-22::gfp]* (Okkema et al., 1997), OK0041 *cuIs3[8xB207::Δpes-10::gfp]* (Okkema and Fire, 1994), VC763 *nfya-1(ok1174) X* (provided by International *C. elegans* Gene Knockout Consortium), OK0971 *cuIs23; nfya-1(ok1174) X,* OK0660 *tbx-2(bx59)* (Huber et al., 2013), OK0661 *nfyb-1(cu13) II; tbx-2(bx59) III*, OK0814 *nfyb-1(cu13) II*, OK0854 *cuEx683[Ptbx-2^{prox}::gfp]*, OK0855 *cuEx684[Ptbx-2^{prox}::gfp]*, OK0851 *cuEx680[Ptbx-2^{dist}::gfp]*, and OK0843 *cuEx672[Ptbx-2$^{prox+dist}$::gfp]*.

The chromosomally integrated *Ptbx-2::gfp* reporter OK0592 [*cuIs23 III*] bearing pOK206.33 + pRF4 was derived from OK0551 *cuEx482* following EMS mutagenesis and was outcrossed twice to N2 (Anderson, 1995; Roy Chowdhuri et al., 2006). The *tbx-2* promoter fragment in pOK206.33 contains 3787 bp upstream of the *tbx-2* ATG (bp 5,139,963 -5,136,174 of LGIII, accession NC_003281.10) inserted in-frame into the *gfp* plasmid pOK95.69 (provided by A. Fire, Stanford University). For crossing *cuIs23* into mutant backgrounds, multiplexed, single-worm PCR was used to genotype animals for *rrf-3(pk1426)* using primers PO848 [TTCTGCGATTGCGATTGG], PO849 [GGTATTTATTGCTTCCTGCCAC], and PO850 [GTCGTCTCTCTCCATTCCAGC]; *nfya-1(ok1174)* using primers PO962 [AAAATGAATGGAGCGTCGAG]. PO963 [TGGACGTGGTCGTGATATTG], and PO964 [TACTTTCCAGCAGCCGAATC], and *nfyb-1(cu13)* using primers PO1381 [CAATTTTTGAGCTGGAAGCC], PO1382 [TTACTGCAGGTAAAACCGGG], and PO1199 [CAGAAGCCGAAATTAAAAGCC] under conditions similar to those previously described (Beaster-Jones and Okkema, 2004). *tbx-2(bx59)* disrupts a BstCI restriction site, and animals were genotyped for this allele as described previously (Huber et al., 2013).

General methods for nucleic acid manipulations and plasmid construction

Standard methods were used to manipulate plasmid DNAs and oligonucleotides (Ausubel, 1990), and all plasmid sequences are available from the authors. CCAAT-boxes at -3609 and -245 (bp 5139738-5139743 and 5136375-5136379, respectively, in LGIII, accession NC_003281.10) using the Stratagene QuikChange II Kit in the *Ptbx-2::gfp* plasmid pOK206.33 (Roy Chowdhuri et al., 2006) to produce *Ptbx-2^{prox}::gfp* plasmid pOK255.07 and *Ptbx-2^{dist}::gfp* plasmid pOK255.08, respectively, and the double mutant *Ptbx-2$^{prox+dist}$::gfp* plasmid pOK270.06 was constructed from these plasmids using standard ligation. All plasmids were sequenced to verify the presence of the mutations.

RNAi analyses

The RNAi feeding screen was modified from Kamath and Ahringer (2003). *E. coli* strains for feeding RNAi of transcription factor genes were recovered from the *C. elegans* RNAi feeding library and the Transcription Factor sub-set (MRC Geneservice) (Kamath and Ahringer, 2003; Reece-Hoyes et al., 2005), and feeding RNAi was performed on OK0592 *cuIs23[Ptbx-2::gfp] III* or in the RNAi hypersensitive strain OK0969 *rrf-3(pk1426) II; cuIs23 III* at 20°C or 16°C, respectively. Briefly, L4 hermaphrodites were plated seeded RNAi plates for 24 hr transferred to freshly seeded RNAi plates, and removed after 1–3 days. The F1 progeny were scored for lethality and morphological abnormalities, and for alterations in *Ptbx-2::gfp* expression using a Leica MZ FLIII fluorescence dissecting microscope and a Zeiss Axioskop compound microscope.

RNAi analyses using injection of double-stranded RNA were performed essentially as previously described (Fire et al., 1998). dsRNA produced by vitro transcription (Ambion) of cDNAs from *nfya-1* (yk468e11/pOK296.01), *nfya-2* (yk635h6/pOK296.03), *nfyb-1*

(yk401d5/pOK296.05), *nfyc-1* (yk232h5/pOK296.07), and *tbx-2* (yk112c4/pOK165.07) provided by Y. Kohara (National Institute of Genetics, Japan) was injected into *Ptbx-2::gfp* containing strains at approximately 200–500 ng/μl at 20°C. Injected animals were transferred to fresh plates every 12 hours, and animals laid 24–48 hours post injection were scored for altered *Ptbx-2::gfp* expression.

Quantification of mRNA levels using real-time PCR

Synchronized N2 and *nfya-1(ok1174)* L1 larvae were prepared by bleaching mixed-stage animals and allowing the isolated embryos to grow 12 hr on OP50 seeded NGM plates at 20°C (Lewis and Fleming, 1995). Total RNA was isolated using TRIzol (Invitrogen), and purified using the RNeasy Mini Kit (Qiagen) and the TURBO DNA-free kit (Ambion) according to manufacturers' instructions. Reverse transcription and real-time PCR was performed using 100 ng total RNA and the Power SYBR Green RNA-to-Ct 1 step kit (Invitrogen) in an ABI 7500FAST PCR thermocycler (Applied Biosystems). Three to four replicates were performed for each RNA sample, and three independent RNA samples were assayed for each genotype. Exonic primers that spanned introns in *tbx-2* (PO1048 [TCAAAACGAGAAGGTGACGG]; PO1072 [ATGTGTGGGTAGTGAAGCGG]) and *ama-1* (PO1061 [AGGCGAAGGATGTGTTGTG]; PO1062 [TCACCGTGTTCTTTGGGTC]) were used, and the level of *tbx-2* expression relative to that of *ama-1* was compared in N2 and *nfya-1(ok1174)* animals using the $2^{-\mathrm{l}\Delta\Delta\mathrm{Ct}}$ method (Livak and Schmittgen, 2001).

Isolation, mapping and identification of *nfyb-1(cu13)*

cu13 arose spontaneously in the *tbx-2(bx59)* background and was outcrossed with wild-type N2. Self-progeny of *cu13/+; tbx-2(bx59)/+* heterozygotes were recovered at 16°C. Five percent of these isolated strains exhibited highly penetrant L1 arrest when shifted to 25°C (n=119). Animals homozygous for unlinked alleles are expected to arise at a frequency of 6.25%, indicating *cu13* is an unlinked, recessive enhancer of *tbx-2(bx59)*. SNP mapping using the polymorphic strain CB4856 indicated *cu13* maps on LGII left of pkP2103 (LGII bp 3,828,608) (Davis et al., 2005). To outcross *cu13* from the *tbx-2(bx59)* background, progeny of *cu13 + +/+ dpy-10(e128) unc-4(e120); tbx-2(bx59)/+* animals were picked to individual plates and screened for animals that lacked the *tbx-2(bx59)* allele by PCR and that segregated 100% non-Dpy non-Unc progeny. This strain OK0814 was verified to be homozygous for *cu13* by crossing back into *tbx-2(bx59)* and recovering animals exhibiting the enhanced L1 arrest phenotype.

Illumina paired-end sequencing was used to sequence genomic DNA from OK0660 [*tbx-2(bx59)*], OK0661 [*cu13; tbx-2(bx59)*], OK0814 [*cu13*], and N2, and we obtained 59- to 79-fold coverage for each genome. Sequences were aligned to the reference *C. elegans* genome (WS220; www.wormbase.org) using Bowtie 2 (Langmead and Salzberg, 2012), and SNP/INDEL variants were identified using the SAMtools package (Li et al., 2009). Variants were annotated with ANNOVAR software (Wang et al., 2010) using genome annotatons retrieved from the USCS Genome Broswer (ce10/WS220). Variants were compared between genomes using Microsoft Access, and variants and the underlying sequence reads were visualized using the Integrative Genomics Viewer (IGV) (Thorvaldsdottir et al., 2012). No SNP/INDELs affecting coding sequences on LGII specifically in the OK0661 and OK0814 genomes were found. To identify deletions, base calls for LGII output from Bowtie 2 SAM files were directly entered into a Microsoft Access database, and regions of low sequence coverage in the OK0661 and OK0814 were manually examined using the IGV. *cu13* is a 5116 bp deletion/1 bp insertion (A/T) that removes bp 460,880-465,995 of LGII (WS220).

Microscopy

Worms were visualized using Zeiss Axioskop and AxioImager microscopes equipped for DIC and fluorescence microscopy, and images were captured using an Axiocam MRm camera and AxioVision or Zen 2011 software.

Results

nfyc-1 negatively regulates *Ptbx-2::gfp* expression

tbx-2 is expressed in a dynamic pattern throughout the *C. elegans* life cycle, and we are interested in identifying mechanisms that regulate *tbx-2* expression. We have previously described a *tbx-2::gfp* promoter fusion that contains approximately 3.7 kb of *tbx-2* 5′-flanking DNA fused to the *gfp* coding sequence just downstream of the *tbx-2* translational initiation site, and we subsequently refer to it as *Ptbx-2::gfp* (Figure 1A) (Roy Chowdhuri et al., 2006). In embryos, *Ptbx-2::gfp* was expressed in pharyngeal precursors beginning at the 100–200 cell stage, and GFP remained detectable in the pharynx until near hatching (Figure 1B,C). During larval and adult stages *Ptbx-2::gfp* was strongly expressed in several neurons in the head and occasionally neurons in the tail, but it was not expressed in the pharynx or other tissues (Figure 1D–G).

To identify mechanisms regulating *tbx-2* expression, we used feeding RNAi to knock down expression of transcription factor genes in strains containing a chromosomally integrated *Ptbx-2::gfp* reporter and screened for changes in GFP expression (see Materials and Methods). We examined 725 of the 934 predicted transcription factor genes in the *C. elegans* genome (Supplemental Table 1) (Reinke et al., 2013) and found that RNAi knock down of two genes, *nfyc-1* and *tbx-2* itself, resulted in ectopic *Ptbx-2::gfp* expression in the gut. By comparison, no genes were identified whose knockdown eliminated *Ptbx-2::gfp* expression. In the remainder of this paper we focus on characterizing the role of *nfyc-1* and related genes in *tbx-2* regulation.

To more closely examine the effect of *nfyc-1* knockdown, we examined *Ptbx-2::gfp* expression in *nyfc-1(RNAi)* animals produced by injecting double-stranded *nfyc-1* RNA (Fire et al., 1998). These *nfyc-1(RNAi)* animals exhibited ectopic GFP expression throughout the gut, as observed in the feeding RNAi screen, as well as in the seam cells, a group of hypodermal blast cells arranged as longitudinal rows on the lateral surface of the worm (Figure 2A,B). In both tissues, ectopic *Ptbx-2::gfp* expression was limited to L4 and adult animals. These results suggest NFYC-1 represses *tbx-2* promoter activity in seam and gut cells.

The NF-Y complex negatively regulates *tbx-2* expression

nfyc-1 encodes the C-subunit of the conserved heterotrimeric transcription factor NF-Y. In mammals, NF-Y is a ubiquitously expressed complex comprised of A, B, and C subunits (Mantovani, 1999). In *C. elegans*, there are two distinct NF-Y complexes that share B and C subunits encoded by the *nfyb-1* and *nfyc-1* genes, respectively, but they contain different A subunits encoded by either *nfya-1* or *nfya-2* (Deng et al., 2007). To ask if either NF-Y complex represses *tbx-2* expression, we examined *Ptbx-2::gfp* in animals in which other NF-Y subunits were individually knocked down by RNAi. As in *nfyc-1(RNAi)* animals, *Ptbx-2::gfp* was ectopically expressed in seam cells and gut of *nfya-1(RNAi)* and *nfyb-1(RNAi)* animals in late larval stages through adulthood (Figure 2C–F). In contrast, *nfya-2(RNAi)* animals exhibited only infrequent and faint expression in seam cells of the head, suggesting NFYA-2 plays only a minor role in repressing *Ptbx-2::gfp* expression (Figure 3). As a control to ask if NF-Y knockdown specifically derepressed *Ptbx-2::gfp*, we examined expression of two additional pharyngeal markers *ceh-22::gfp* and a *myo-2*

enhancer*::gfp* and found their expression was unaffected by *nfyb-1(RNAi)* (Okkema and Fire, 1994; Okkema et al., 1997) (data not shown). Together these results indicate the NF-Y complex containing NFYA-1 specifically represses *Ptbx-2::gfp* expression.

To further assess the specificity of the loss of NF-Y phenotype, we examined *Ptbx-2::gfp* expression in an *nfya-1* null mutant (Figure 4A, B). *nfya-1(ok1174)* homozygotes were viable but exhibited slow growth and male mating defects consistent with the phenotypes in previously described *nfya-1* mutants (Deng et al., 2007). Just as when NF-Y components were knocked down by RNAi, *nfya-1(ok1174)* mutants ectopically expressed *Ptbx-2::gfp* in seam cells and the gut (Figure 2G, H; Table 1). However, unlike our RNAi experiments, ectopic *Ptbx-2::gfp* expression in the gut of *nfya-1(ok1174)* mutants was detectable as early as the L1 stage (Figure 5A,B). These results suggest that NF-Y regulation of *tbx-2* expression occurs throughout larval development, and that *nfya-1(RNAi)* is less effective in knocking out activity of the NF-Y complex than the null mutant.

Two CCAAT-boxes in the *tbx-2* promoter are important for *tbx-2* regulation

NF-Y binds CCAAT-boxes, which are common eukaryotic regulatory sites typically found in promoters approximately 60 to 100 bp upstream of the transcriptional start site (Mantovani, 1999). While the transcriptional start site for *tbx-2* is not known, the *tbx-2* promoter contains two CCAAT-boxes located at 3566 bp and 203 bp upstream of the translational start codon, and we refer to these as the distal and proximal sites, respectively (Figure 1A). To ask if these sites regulate *tbx-2* promoter activity, we examined the effect of mutating them on *Ptbx-2::gfp* expression in multiple independent transgenic lines. A mutation of the proximal CCAAT-box (*Ptbx-2*^{prox}*::gfp*) resulted in very frequent ectopic GFP expression in seam cells and gut (Figure 6; Table 1). In comparison, a mutation of the distal CCAAT-box (*Ptbx-2*^{dist}*::gfp*) resulted in much less frequent ectopic GFP expression (Figure 6; Table 1). However *Ptbx-2*^{dist}*::gfp* was expressed more frequently in seam cells and more widely throughout the gut than wild-type *Ptbx-2::gfp*, for which the occasional gut expression we observed was limited to posterior cells. A double mutant of both CCAAT-boxes (*Ptbx-2*$^{prox+dist}$*::gfp*) was expressed similarly to the *Ptbx-2*^{prox}*::gfp* single mutant in seam cells, but it was expressed less frequently in the gut than *Ptbx-2*^{prox}*::gfp* (Table 1). We conclude that the proximal CCAAT-box is the major site repressing *Ptbx-2::gfp* in the seam cells and gut. The distal site also plays a minor role in repressing *Ptbx-2::gfp*, but it may have an additional activating role, because the double CCAAT-box mutant had less frequent expression than the proximal mutant alone. Because both loss of NF-Y function and these CCAAT-box mutations lead to ectopic *Ptbx-2::gfp* expression in identical patterns, it is likely that NF-Y directly represses the *tbx-2* promoter.

NF-Y represses endogenous *tbx-2* expression

While NF-Y clearly represses *Ptbx-2::gfp*, we wanted to know if it regulates expression of the endogenous *tbx-2* gene. *tbx-2* mRNA levels in wild-type and *nfya-1(ok1174)* mutants were compared using quantitative real-time PCR. Because *nfya-1(ok1174)* larvae grow more slowly than wild type, we used total RNA isolated from synchronized L1 animals to avoid differences in mutant and wild-type populations resulting from these different larval growth rates. Ectopic *Ptbx-2::gfp* expression was first detectable in the guts of *nfya-1(ok1174)* L1s (Figure 5B), and we hypothesized that ectopic expression of endogenous *tbx-2* expression would similarly begin at this stage. We found *nfya-1(ok1174)* mutants over-expressed *tbx-2* 1.40±0.07 fold relative to wild-type. While this difference is relatively small, it is highly significant (p=0.005), and it likely reflects the onset of ectopic *tbx-2* expression. These results argue that NF-Y represses expression of endogenous *tbx-2* similarly to *Ptbx-2::gfp*.

An *nfyb-1* mutation enhances the phenotype of a *tbx-2* mutant

tbx-2(bx59) is a temperature sensitive mutant exhibiting partially penetrant lethality and pharyngeal defects, and it provides a compromised genetic background that can be used to identify genes affecting *tbx-2* activity (Huber et al., 2013). We isolated a spontaneous mutation called *cu13* that enhances *tbx-2(bx59)* lethality and, using SNP mapping and whole genome sequencing, we identified *cu13* as an allele of *nfyb-1* (Figure 4C) (Davis et al., 2005; Sarin et al., 2008). *nfyb-1(cu13)* is a 5116 bp deletion/1 bp insertion that removes the last 5 exons of this gene. Over 50% of the *nfyb-1* coding sequence is deleted, and this deletion is predicted to truncate the NFYB-1 protein downstream of the histone fold domain (HFD) involved in DNA binding (Figure 4D). The endpoints of the *cu13* deletion are located within tandem repeat sequences that are enriched in this region (Wormbase WS230), and these repeats likely contributed to formation of this deletion.

We out-crossed *nfyb-1(cu13)* from the *tbx-2(bx59)* mutant background and found that these *nfyb-1(cu13)* single mutants ectopically expressed *Ptbx-2::gfp* in a pattern identical to *nfya-1(ok1174)* mutants. Nearly all *nfyb-1(cu13)* animals expressed *Ptbx-2::gfp* in seam cells and gut at the L4 and adult stages (Figure 2I,J; Table 1). As in *nfya-1* mutants, ectopic *Ptbx-2::gfp* expression was detectable in the gut as early L1 stage (Figure 5C), and it increased in strength and frequency the seam cells through larval development.

This unexpected finding of an *nfyb-1* mutation as an enhancer of the *tbx-2* mutant phenotype indicates NF-Y regulation is crucial for *tbx-2* expression *in vivo*, and we compared the phenotype of *nfyb-1(cu13); tbx-2(bx59)* double mutants with those of *tbx-2(bx59)* and *nfyb-1(cu13)* single mutants. When *tbx-2(bx59)* hermaphrodites were shifted to the non-permissive temperature, their progeny exhibited a partially penetrant L1 arrest phenotype (Huber et al., 2013), and this phenotype was strongly enhanced by *nfyb-1(cu13)*. Forty-four percent of *tbx-2(bx59)* animals arrested as L1 larvae, and most of the remaining animals grew to adulthood (Table 2; Table 3). In comparison, all *nfyb-1(cu13); tbx-2(bx59)* double mutants arrested as L1 animals, and none of these animals grew to adults. As with other NF-Y mutants (Deng et al., 2007), no L1 arrest was observed in *nfyb-1(cu13)* single mutants, indicating the lethality in the double mutants results from enhancement of the *tbx-2(bx59)* phenotype.

tbx-2(bx59) single mutants exhibit variable pharyngeal defects (Huber et al., 2013). Nineteen percent of these animals have a severe phenotype with a clear loss of ABa-derived pharynx, similar to that observed in *tbx-2* null mutants (Roy Chowdhuri et al., 2006). The remaining animals exhibit either moderate morphological defects (56%) or normal pharyngeal morphology (25%) (Huber et al., 2013). We expected that the enhanced L1 arrest observed in *nfyb-1(cu13); tbx-2(bx59)* double mutants would result from increased severity of the pharyngeal defect. However, we did not find any *nfyb-1(cu13); tbx-2(bx59)* animals exhibiting the severe phenotype and a loss of ABa-derived pharynx. Rather *nfyb-1(cu13); tbx-2(bx59)* animals exhibited a moderate morphological defect in the pharynx (81%, n=28) at a frequency higher than *tbx-2(bx59)* single mutants (54%, n=26) (Figure 7). The fact that these pharyngeal defects were not enhanced indicates *nfyb-1(cu13); tbx-2(bx59)* lethality did not result from eliminating *tbx-2* expression in the pharynx.

Loss of *nfyb-1* and *tbx-2* function causes enhanced lethality

The synergistic phenotypes observed in *nfyb-1(cu13); tbx-2(bx59)* double mutants argue that both of these genes function in the same pathway *in vivo* [see (Herman and Yochem, 2005)]. However, we do not know whether these individual mutations result in gain- or loss-of-function. The region deleted in *nfyb-1(cu13)* encodes no recognizable functional domains, and this allele could produce a deleted protein that retains the HFD (Figure 4D).

tbx-2(bx59) is a missense mutation that substitutes a conserved histidine residue in the dimerization domain of the TBX-2 DNA-binding domain with tyrosine (H154Y; accession CC69847) (K. Chow, personal communication) (Huber et al., 2013), and mutations affecting nearby residues in the dimerization domain of human TBX1 are gain-of-function mutations that underlie some cases of DiGeorge and velocardiofacial syndromes (Zweier et al., 2007). To examine the nature of *nfyb-1(cu13)* and *tbx-2(bx59)* mutations we used RNAi to knock down activity of *nfyb-1* or *tbx-2* in either *tbx-2(bx59)* or *nfyb-1(cu13)* mutants, respectively, and in the *nfyb-1(cu13); tbx-2(bx59)* double mutant.

We predicted that if *nfyb-1(cu13)* was a loss-of-function allele, *nfyb-1(RNAi)* should increase lethality in *tbx-2(bx59)* mutants, whereas, if it was a gain-of-function allele, *nfyb-1(RNAi)* should suppress larval lethality in *nfyb-1(cu13); tbx-2(bx59)* double mutants. We observed a strongly enhanced larval lethal phenotype in *nfyb-1(RNAi); tbx-2(bx59)* double mutants compared to *tbx-2(bx59)* or *nfyb-1(RNAi)* alone, and we saw no suppression of the larval lethal phenotype in *nfyb-1(RNAi); nfyb-1(cu13); tbx-2(bx59)* animals (Table 3). Thus we conclude that *nfyb-1(cu13)* is a loss-of-function allele, and that loss of *nfyb-1* enhances the *tbx-2(bx59)* phenotype.

We similarly predicted that if *tbx-2(bx59)* was a loss-of-function allele, *tbx-2(RNAi)* would enhance the lethality of *nfyb-1(cu13)* mutants, while, if it was a gain-of-function allele, *tbx-2(RNAi)* would suppress lethality of *nfyb-1(cu13); tbx-2(bx59)* double mutants. Again we found that *tbx-2(RNAi)* strongly enhanced the larval lethality in the *nfyb-1(cu13)* background, while we saw no suppression of lethality in *tbx-2(RNAi); nfyb-1(cu13); tbx-2(bx59)* animals (Table 4). We also asked whether one functional allele of *tbx-2* was sufficient for viability when *nfyb-1* was reduced. To investigate this, we tested if *nfyb-1(RNAi)* would enhance lethality in animals heterozygous for the *tbx-2(ok529)* null mutant. *tbx-2(ok529)* results in recessive L1 lethality, but *tbx-2(ok529)/+* heterozygotes were completely viable (Roy Chowdhuri et al., 2006). We examined the progeny of *tbx-2(ok529)/+* hermaphrodites that had been injected with *nfyb-1* dsRNA, but we did not see a difference in the number of viable, heterozygous *tbx-2(ok529)/+* progeny segregating from animals either injected or non-injected control animals (n=412 and 401, respectively). Together these results indicate that *tbx-2(bx59)* is a hypomorphic, loss-of-function allele, and they suggest that, at the non-permissive temperature, *tbx-2(bx59)* mutants have less TBX-2 activity than *tbx-2(ok529)/+* heterozygotes.

Discussion

Here we show that NF-Y is a tissue- and stage-specific repressor of *C. elegans tbx-2* gene expression. Loss of NF-Y leads to ectopic expression of *Ptbx-2::gfp* in the seam cells and gut of larvae and adults and increased expression of endogenous *tbx-2*. NF-Y repression of *tbx-2* is likely to be direct, as both loss of NF-Y and mutation of CCAAT-boxes within the *tbx-2* promoter lead to nearly identical patterns of ectopic *Ptbx-2::gfp* expression. Finally the fact that an *nfyb-1* mutation enhances the lethal phenotype of a hypomorphic *tbx-2* mutant argues that NF-Y repression is crucial for normal *tbx-2* function *in vivo*.

C. elegans NF-Y represses transcription

CCAAT-boxes are common eukaryotic regulatory sequences, found near the transcription start site in approximately 12% of human promoters (Dolfini et al., 2009). The conserved NF-Y complex is the major CCAAT-box binding factor and NF-Y binding is typically associated with transcriptional activation (Mantovani, 1999). However, examples of transcriptional repression are also known (Buschlen et al., 2003; Ceribelli et al., 2008). Genome wide characterization of NF-Y targets has not been carried out in invertebrate model organisms. However, characterization of the Drosophila p53 and sevenless genes

indicates that NF-Y can function as an activator in this organism (Tue et al., 2011; Yoshioka et al., 2012).

C. elegans NF-Y has only begun to be characterized, but, in the few known examples, it functions as a transcriptional repressor. As we have found for *tbx-2*, expression of the Hox genes *egl-5* and *mab-5* is repressed by NF-Y (Deng et al., 2007). This repression is tissue-specific, and loss of *nfya-1* or mutation of a CCAAT-box in the *egl-5* promoter leads to ectopic *egl-5::gfp* expression in seam cells and elsewhere in the hypodermis. This incomplete overlap with the tissues where *Ptbx-2::gfp* is repressed suggests the specific mechanisms by which NF-Y represses *tbx-2* and *egl-5* are distinct. Indeed, NF-Y interacts with the MES-2/MES-6 PcG complex to repress *egl-5*, but we have not observed a role for MES-2/MES-6 in repressing *tbx-2* expression.

At this point, it is not known what other genes NF-Y regulates in *C. elegans*, or whether *C. elegans* NF-Y can activate as well as repress transcription. Indeed, our work suggests the distal CCAAT-box in *Ptbx-2::gfp* weakly activates expression, implying NF-Y can both repress and activate transcription. While *C. elegans* NF-Y subunits are conserved with those in mammals, they do have some interesting differences that might affect their activities. For example, *C. elegans* NFYC-1 lacks a glutamine-rich transcriptional activation domain found in mammalian NF-YC subunits (Coustry et al., 1996; Deng et al., 2007). This difference suggests that *C. elegans* NF-Y may not be as strong an activator as mammalian NF-Y, or that it activates transcription with co-activators, as has been observed with the yeast HAP complex (Olesen and Guarente, 1990). In another example, *C. elegans* NFYA-1 contains a G to D substitution converting the highly conserved GXGGRF motif to GXDGRF. In human NF-Y, this affected G directly contacts the a base in the CCAAT box (Nardini et al., 2013), and the G to D substitution could affect NF-Y binding affinity or specificity. Interestingly NFYA-2 contains an XGGRF more similar to the motif in human NF-YA, and these differences suggest the NF-Y complexes containing NFYA-1 or NFYA-2 may target different sites in the genome. Additional work will be necessary to identify more *C. elegans* NF-Y targets, and determine if NF-Y can activate transcription in this organism.

Ptbx-2::gfp expression is specifically repressed in seam cells and the gut

C. elegans NF-Y is broadly expressed, yet loss of NF-Y regulation results in *Ptbx-2::gfp* expression only in the seam cells and gut. This suggests that the *tbx-2* promoter is poised for expression in these tissues. Seam cells and gut are derived from different germ layers and are lineally unrelated (Sulston et al., 1983). However, these tissues share the common feature that they undergo DNA replication each larval stage. Seam cells replicate DNA and divide in a stem cell-like pattern near the end of each stage, producing one differentiated daughter and one seam cell daughter (Joshi et al., 2010), and gut cells replicate DNA and undergo either a nuclear division or endoreduplication at each larval stage (Hedgecock and White, 1985). Perhaps *C. elegans tbx-2* is primed for low level or transient expression in seam cells and gut to promote DNA replication, but NF-Y represses expression to undetectable levels under most circumstances.

Mammalian *TBX2* and *TBX3* are orthologs of *C. elegans tbx-2*, and they function as regulators of cell cycle progression and as inhibitors of cellular senescence [reviewed in (Lu et al., 2010)]. Interestingly, they are both believed to be activated by NF-Y (Smith et al., 2011; Teng et al., 2008). Although this regulation is different from what we have observed for *C. elegans tbx-2*, it shows NF-Y is a regulator of T-box gene expression in very different species.

NF-Y affects *tbx-2* activity *in vivo*

Our finding that the spontaneous mutant *nfyb-1(cu13)* enhances lethality of *tbx-2(bx59)* demonstrates NF-Y plays an important role in regulating *tbx-2* activity *in vivo*. However the mechanism by which loss of NF-Y regulation enhances *tbx-2(bx59)* lethality is unclear. Because *nfyb-1(cu13)* is viable in a *tbx-2(+)* background, simple loss of repression resulting in expression of *tbx-2* in seam cells and gut would not explain the enhanced lethality in *nfyb-1(cu13); tbx-2(bx59)* double mutants. One possibility is that NF-Y and TBX-2 have partially overlapping functions regulating other downstream genes, and loss of both TBX-2 and NF-Y regulation leads to lethality in *nfyb-1(cu13); tbx-2(bx59)* mutants. Alternatively, mutations in *nfyb-1* and *tbx-2* may lead to even higher levels of *tbx-2* overexpression than *nfyb-1* mutants alone. We identified *tbx-2* in our RNAi screen as a potential negative regulator of *Ptbx-2::gfp*, and we are currently investigating whether NF-Y and TBX-2 function in parallel to repress the *tbx-2* promoter.

Like our observations with *nfyb-1*, other genetic modifiers of T-box gene mutants reveal important mechanisms regulating T-box gene expression and activity. *C. elegans* TBX-2 is post-translationally modified by SUMOylation, and we have shown that reducing SUMOylation enhances the *tbx-2(bx59)* phenotype (Huber et al., 2013). Likewise reducing the mouse MOZ histone acetyltransferase enhances defects resulting from haploinsufficiency of *Tbx1*, and it is suggested that the epigenetic regulatory state of human *TBX1* contributes to the variable severity of symptoms observed in DiGeorge syndrome patients (Voss et al., 2012). Characterization of additional modifiers of T-box gene mutations will likely continue to provide novel insights into T-box factor function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Abrahams A, Parker MI, Prince S. The T-box transcription factor Tbx2: its role in development and possible implication in cancer. IUBMB life. 2010; 62:92–102. [PubMed: 19960541]

Anderson, P. Mutagenesis. In: Epstein, HF.; Shakes, DC., editors. Caenorhabditis elegans: Modern Biological Analysis of an Organism. Academic Press; San Diego, CA: 1995. p. 31-58.

Ausubel, FM. Current protocols in molecular biology. Greene Pub. Associates and Wiley-Interscience : J. Wiley; New York: 1990.

Beaster-Jones L, Okkema PG. DNA binding and in vivo function of C. elegans PEB-1 require a conserved FLYWCH motif. J Mol Biol. 2004; 339:695–706. [PubMed: 15165844]

Buschlen S, Amillet JM, Guiard B, Fournier A, Marcireau C, Bolotin-Fukuhara M. The S. Cerevisiae HAP complex, a key regulator of mitochondrial function, coordinates nuclear and mitochondrial gene expression. Comparative and functional genomics. 2003; 4:37–46. [PubMed: 18629096]

Ceribelli M, Dolfini D, Merico D, Gatta R, Vigano AM, Pavesi G, Mantovani R. The histone-like NF-Y is a bifunctional transcription factor. Molecular and cellular biology. 2008; 28:2047–2058. [PubMed: 18212061]

Coustry F, Maity SN, Sinha S, de Crombrugghe B. The transcriptional activity of the CCAAT-binding factor CBF is mediated by two distinct activation domains, one in the CBF-B subunit and the other in the CBF-C subunit. The Journal of biological chemistry. 1996; 271:14485–14491. [PubMed: 8662945]

Davis MW, Hammarlund M, Harrach T, Hullett P, Olsen S, Jorgensen EM. Rapid single nucleotide polymorphism mapping in C. elegans. BMC Genomics. 2005; 6:118. [PubMed: 16156901]

Deng H, Sun Y, Zhang Y, Luo X, Hou W, Yan L, Chen Y, Tian E, Han J, Zhang H. Transcription factor NFY globally represses the expression of the C. elegans Hox gene Abdominal-B homolog egl-5. Dev Biol. 2007; 308:583–592. [PubMed: 17574230]

Dolfini D, Zambelli F, Pavesi G, Mantovani R. A perspective of promoter architecture from the CCAAT box. Cell Cycle. 2009; 8:4127–4137. [PubMed: 19946211]

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998; 391:806–811. [PubMed: 9486653]

Hedgecock EM, White JG. Polyploid tissues in the nematode Caenorhabditis elegans. Dev Biol. 1985; 107:128–133. [PubMed: 2578115]

Herman RK, Yochem J. Genetic enhancers. WormBook : the online review of C. elegans biology. 2005:1–11.

Huber P, Crum T, Clary LM, Ronan T, Packard AV, Okkema PG. Function of the C. elegans T-box factor TBX-2 depends on SUMOylation. Cellular and molecular life sciences : CMLS. 2013

Joshi PM, Riddle MR, Djabrayan NJ, Rothman JH. Caenorhabditis elegans as a model for stem cell biology. Dev Dyn. 2010; 239:1539–1554. [PubMed: 20419785]

Kamath RS, Ahringer J. Genome-wide RNAi screening in Caenorhabditis elegans. Methods. 2003; 30:313–321. [PubMed: 12828945]

Kohara Y. Systematic analysis of gene expression of the C. elegans genome. Tanpakushitsu Kakusan Koso. 2001; 46:2425–2431. [PubMed: 11802405]

Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012; 9:357–359. [PubMed: 22388286]

Lewis, JA.; Fleming, JT. Biology, M.i.C. Caenorhabditis elegans: Modern Biological Analysis of an Organism. Academic Press; San Diego, CA: 1995. Basic Culture Methods; p. 4-30.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009; 25:2078–2079. [PubMed: 19505943]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]

Lu J, Li XP, Dong Q, Kung HF, He ML. TBX2 and TBX3: the special value for anticancer drug targets. Biochimica et biophysica acta. 2010; 1806:268–274. [PubMed: 20624445]

Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene. 1999; 239:15–27. [PubMed: 10571030]

Mantovani R, Li XY, Pessara U, Hooft van Huisjduijnen R, Benoist C, Mathis D. Dominant negative analogs of NF-YA. The Journal of biological chemistry. 1994; 269:20340–20346. [PubMed: 8051128]

Martin N, Benhamed M, Nacerddine K, Demarque MD, van Lohuizen M, Dejean A, Bischof O. Physical and functional interaction between PML and TBX2 in the establishment of cellular senescence. The EMBO journal. 2012; 31:95–109. [PubMed: 22002537]

Mello, C.; Fire, A. DNA Transformation. In: Epstein, HF.; Shakes, DC., editors. Caenorhabditis elegans: Modern Biological Analysis of an Organism. Academic Press; San Diego, CA: 1995. p. 451-482.

Miyahara K, Suzuki N, Ishihara T, Tsuchiya E, Katsura I. TBX2/TBX3 transcriptional factor homologue controls olfactory adaptation in Caenorhabditis elegans. J Neurobiol. 2004; 58:392–402. [PubMed: 14750151]

Naiche LA, Harrelson Z, Kelly RG, Papaioannou VE. T-box genes in vertebrate development. Annu Rev Genet. 2005; 39:219–239. [PubMed: 16285859]

Nardini M, Gnesutta N, Donati G, Gatta R, Forni C, Fossati A, Vonrhein C, Moras D, Romier C, Bolognesi M, Mantovani R. Sequence-specific transcription factor NF-Y displays histone-like DNA binding and H2B-like ubiquitination. Cell. 2013; 152:132–143. [PubMed: 23332751]

Okkema PG, Fire A. The Caenorhabditis elegans NK-2 class homeoprotein CEH-22 is involved in combinatorial activation of gene expression in pharyngeal muscle. Development. 1994; 120:2175–2186. [PubMed: 7925019]

Okkema PG, Ha E, Haun C, Chen W, Fire A. The Caenorhabditis elegans NK-2 homeobox gene ceh-22 activates pharyngeal muscle gene expression in combination with pha-1 and is required for normal pharyngeal development. Development. 1997; 124:3965–3973. [PubMed: 9374394]

Olesen JT, Guarente L. The HAP2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: model for the HAP2/3/4 complex. Genes & development. 1990; 4:1714–1729. [PubMed: 2123465]

Packham EA, Brook JD. T-box genes in human disorders. Hum Mol Genet. 2003; 12(Spec No 1):R37–44. [PubMed: 12668595]

Pocock R, Ahringer J, Mitsch M, Maxwell S, Woollard A. A regulatory network of T-box genes and the even-skipped homologue vab-7 controls patterning and morphogenesis in C. elegans. Development. 2004; 131:2373–2385. [PubMed: 15102704]

Reece-Hoyes JS, Deplancke B, Shingles J, Grove CA, Hope IA, Walhout AJ. A compendium of Caenorhabditis elegans regulatory transcription factors: a resource for mapping transcription regulatory networks. Genome Biol. 2005; 6:R110. [PubMed: 16420670]

Reinke, V.; Krause, M.; Okkema, PG. Community, T.C.e.R. WormBook : the online review of C. elegans biology. WormBook; 2013 Jun 04. Transcriptional regulation of gene expression in C. elegans; p. 1-31.

Renard CA, Labalette C, Armengol C, Cougot D, Wei Y, Cairo S, Pineau P, Neuveut C, de Reynies A, Dejean A, Perret C, Buendia MA. Tbx3 is a downstream target of the Wnt/beta-catenin pathway and a critical mediator of beta-catenin survival functions in liver cancer. Cancer research. 2007; 67:901–910. [PubMed: 17283120]

Roy Chowdhuri S, Crum T, Woollard A, Aslam S, Okkema PG. The T-box factor TBX-2 and the SUMO conjugating enzyme UBC-9 are required for ABa-derived pharyngeal muscle in C. elegans. Dev Biol. 2006; 295:664–677. [PubMed: 16701625]

Sarin S, Prabhu S, O'Meara MM, Pe'er I, Hobert O. Caenorhabditis elegans mutant allele identification by whole-genome sequencing. Nat Methods. 2008; 5:865–867. [PubMed: 18677319]

Showell C, Binder O, Conlon FL. T-box genes in early embryogenesis. Dev Dyn. 2004; 229:201–218. [PubMed: 14699590]

Simmer F, Tijsterman M, Parrish S, Koushika SP, Nonet ML, Fire A, Ahringer J, Plasterk RH. Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. Curr Biol. 2002; 12:1317–1319. [PubMed: 12176360]

Singhvi A, Frank CA, Garriga G. The T-box gene tbx-2, the homeobox gene egl-5 and the asymmetric cell division gene ham-1 specify neural fate in the HSN/PHB lineage. Genetics. 2008; 179:887–898. [PubMed: 18505863]

Smith J, Mowla S, Prince S. Basal transcription of the human TBX3 gene, a key developmental regulator which is overexpressed in several cancers, requires functional NF-Y and Sp1 sites. Gene. 2011; 486:41–46. [PubMed: 21784138]

Smith PA, Mango SE. Role of T-box gene tbx-2 for anterior foregut muscle development in C. elegans. Dev Biol. 2007; 302:25–39. [PubMed: 17005176]

Sulston JE, Schierenberg E, White JG, Thomson JN. The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev Biol. 1983; 100:64–119. [PubMed: 6684600]

Teng H, Parker MI, Prince S. Functional characterization of cis-acting elements involved in basal transcription of the human Tbx2 gene: a new insight into the role of Sp1 in transcriptional regulation. Gene. 2008; 423:8–13. [PubMed: 18640248]

Thorvaldsdottir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform. 2012

Tue NT, Yoshioka Y, Yamaguchi M. NF-Y transcriptionally regulates the Drosophila p53 gene. Gene. 2011; 473:1–7. [PubMed: 21044877]

Voss AK, Vanyai HK, Collin C, Dixon MP, McLennan TJ, Sheikh BN, Scambler P, Thomas T. MOZ regulates the Tbx1 locus, and Moz mutation partially phenocopies DiGeorge syndrome. Developmental cell. 2012; 23:652–663. [PubMed: 22921202]

Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010; 38:e164. [PubMed: 20601685]

Yoshioka Y, Ly LL, Yamaguchi M. Transcription factor NF-Y is involved in differentiation of R7 photoreceptor cell in Drosophila. Biology open. 2012; 1:19–29. [PubMed: 23213364]

Zhang W, Chen H, Wang Y, Yong W, Zhu W, Liu Y, Wagner GR, Payne RM, Field LJ, Xin H, Cai CL, Shou W. Tbx20 transcription factor is a downstream mediator for bone morphogenetic protein-10 in regulating cardiac ventricular wall development and function. The Journal of biological chemistry. 2011; 286:36820–36829. [PubMed: 21890625]

Zweier C, Sticht H, Aydin-Yaylagul I, Campbell CE, Rauch A. Human TBX1 missense mutations cause gain of function resulting in the same phenotype as 22q11.2 deletions. American journal of human genetics. 2007; 80:510–517. [PubMed: 17273972]

Highlights

- The NF-Y complex negatively regulates *C. elegans tbx-2* expression.
- CCAAT-boxes in the *tbx-2* promoter region mediate NF-Y repression.
- An *nfyb-1* mutation was isolated as an enhancer of a *tbx-2* hypomorphic mutant.
- *nfyb-1* and *tbx-2* loss-of-function results in synthetic lethality.

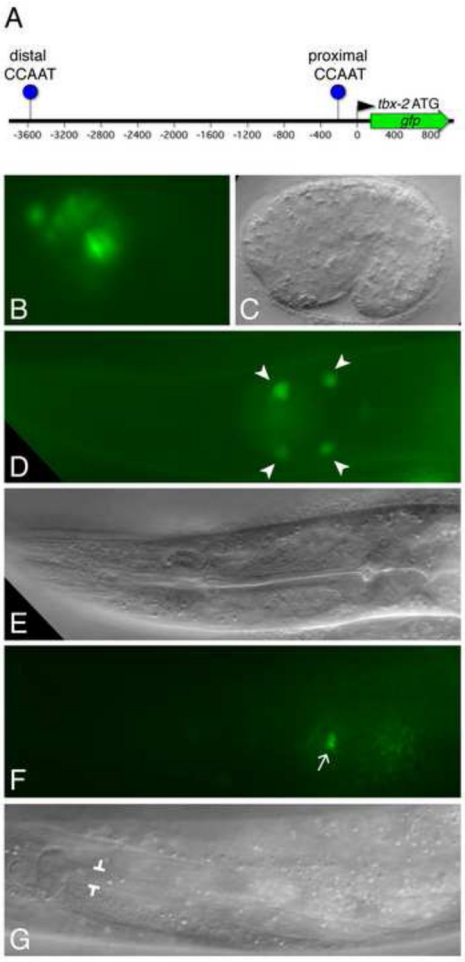

Figure 1. Wild type *Ptbx-2::gfp* expression

A. Schematic diagram of *Ptbx-2::gfp*. Blue dots indicate proximal (-203 bp) and distal (-3566 bp) CCAAT-boxes. B,C. Fluorescence and DIC images of *Ptbx-2::gfp* expression in the pharynx of a 1.5 fold stage embryo (anterior is left). D–G. Fluorescence and DIC images of the head (D,E) and lateral surface (F,G) of young adult animals expressing *Ptbx-2::gfp*. Four GFP expressing neurons outside the pharynx are visible in D (arrowheads), and a region of in focus gut autofluorescence is visible in F (arrow). The width of the seam cells, which do not express GFP in wild-type animals, is indicated by brackets in G.

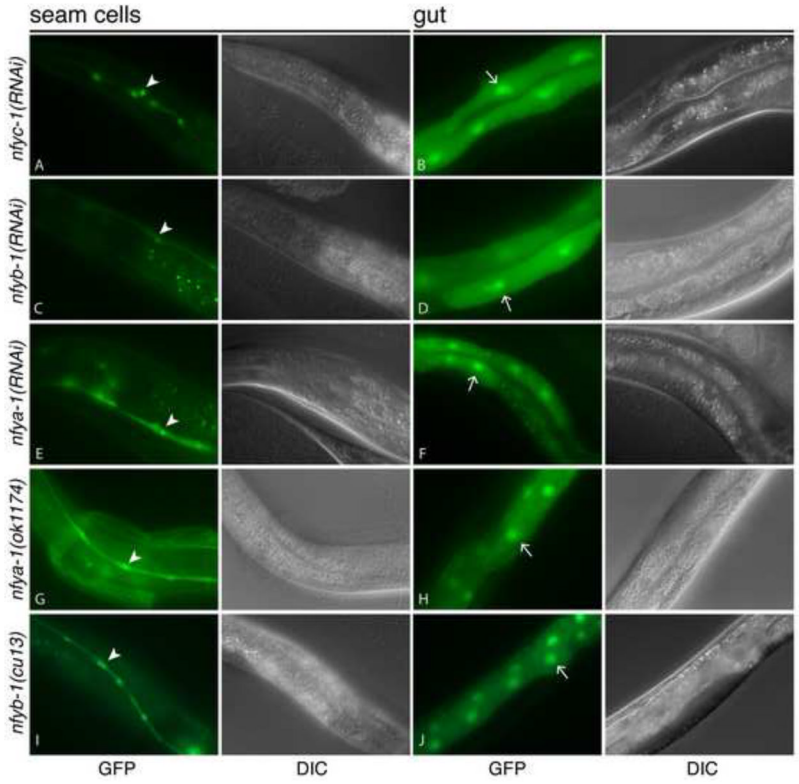

Figure 2. Ectopic *Ptbx-2::gfp* expression in animals with reduced NF-Y

Fluorescence (left) and DIC (right) images of L4 or young adult animals of the indicated genotypes ectopically expressing *Ptbx-2::gfp* in seam cells and gut. GFP accumulates in cell nuclei and cytoplasm. Seam cells are arranged in longitudinal rows on the lateral surface of the worm, and GFP expressing seam cells are marked with arrowheads. The gut is a epithelial tube extending through the central body, and representative GFP containing gut cell nuclei are marked with arrows. For RNAi experiments, C, E, and F are in an *rrf-3(+)* background, while A, B and D are in an RNAi hypersensitive *rrf-3(pk1426)* background. In all cases, ectopic *Ptbx-2::gfp* expression patterns were indistinguishable in wild-type and *rrf-3* mutant backgrounds.

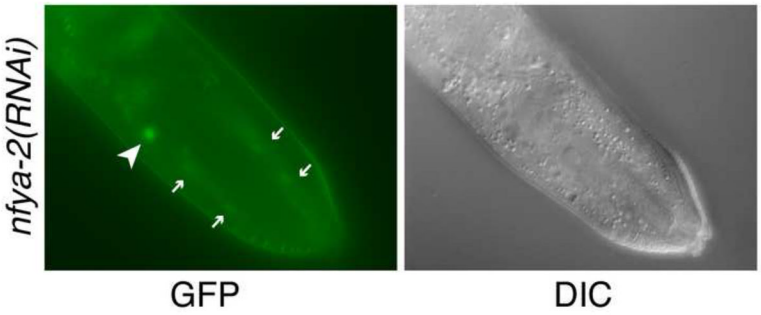

Figure 3. Weak ectopic *Ptbx-2::gfp* expression in *nfya-2(RNAi)*

Fluorescence (left) and DIC (right) images of a young adult animal ectopically expressing *Ptbx-2::gfp* in the seam cells of the head. Arrows indicate faint ectopic *Ptbx-2::gfp* expression in seam cells, and an arrowhead indicates wild-type expression of *Ptbx-2::gfp* in a neuron. This animal is in an *rrf-3(+)* background.

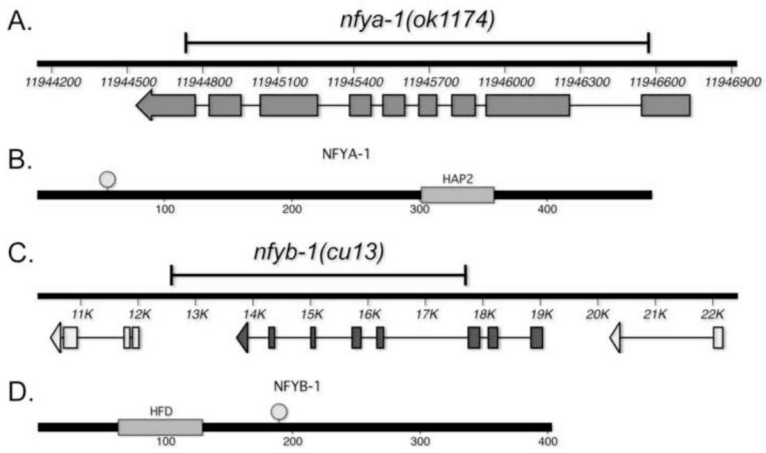

Figure 4. *nfya-1(ok1174)* and *nfyb-1(cu13)* mutations

A. Schematic diagram of the *nfya-1* gene numbered according to accession NC_003284. Dark gray boxes indicate *nfya-1* exons and the direction of transcription. *nfya-1(ok1174)* is an 1837 bp deletion/2 bp insertion (bracket; bp 11944734-11946570) that removes most of the *nfya-1* coding sequence and introduces a frame-shift. B. Schematic diagram of the NFYA-1 protein (accession NP_509999) indicating the HAP2 similarity domain (light gray box), which is necessary for complex formation and DNA-binding (Mantovani et al., 1994). The NFYA-1 protein is predicted to be truncated upstream of the HAP2 domain after residue 55 in this mutant (gray circle). C. Schematic diagram of the *nfyb-1* genomic region numbered according to accession AF025470. Dark gray boxes indicate *nfyb-1* exons and the direction of transcription. *nfyb-1(cu13)* is a 5116 bp deletion/1 bp insertion (bracket; bp 12583-17698) removing 5 exons and a portion of the 3′ UTR region. D. Schematic diagram of the NFYB-1 protein (accession CCD65581) indicating the histone-fold domain (HFD) necessary for DNA binding (light gray box). The NFYB-1 protein is predicted to be truncated downstream of the HFD after residue 189 in this mutant (gray circle).

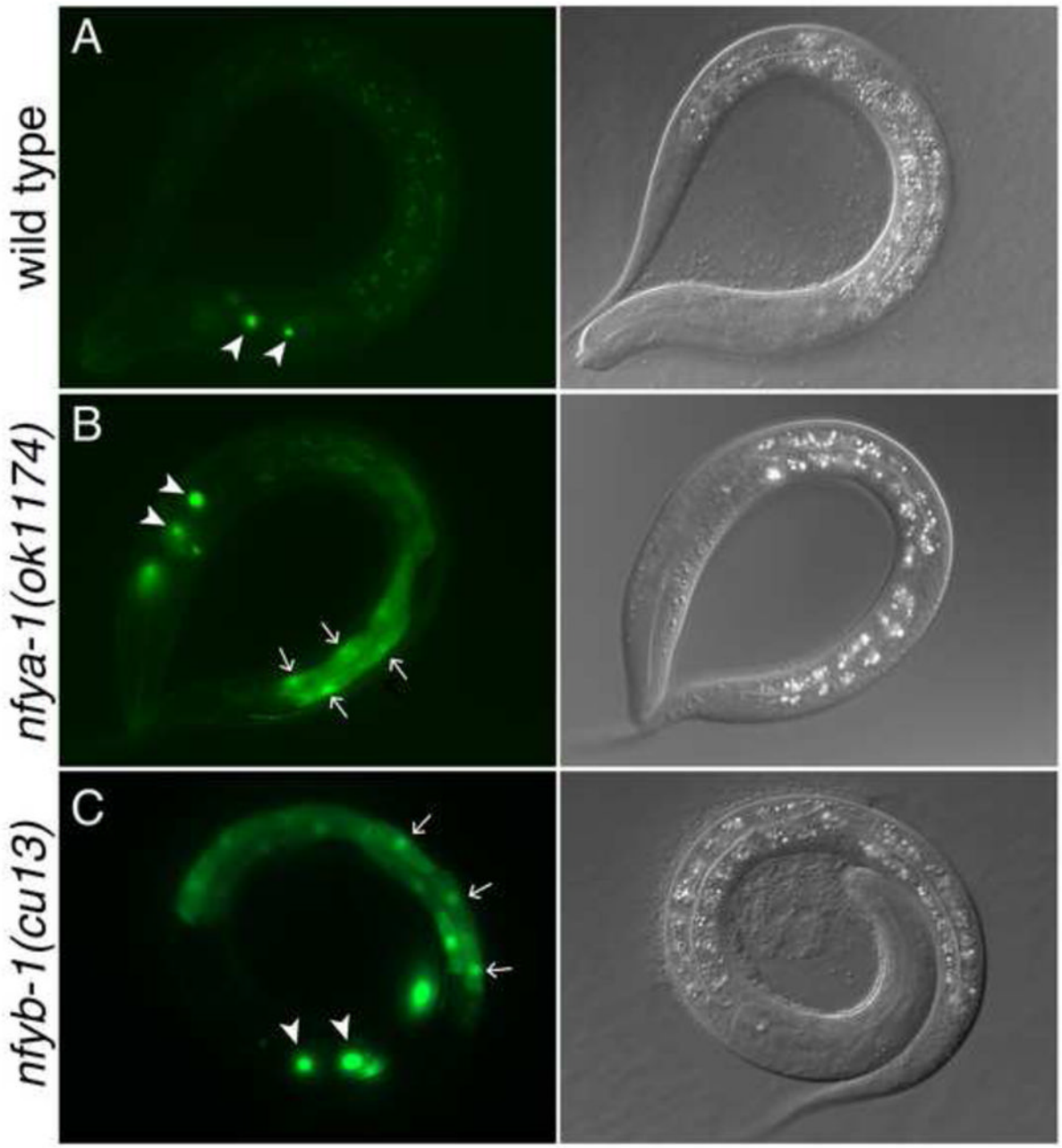

Figure 5. Ectopic *Ptbx-2::gfp* is expressed in early larvae in *nfya-1(ok1174)* and *nfyb-1(cu13)*
Fluorescence (left) and DIC (right) images of wild-type (A), *nfya-1(ok1174)* (B), and *nfyb-1(cu13)* L1 larvae expressing *Ptbx-2::gfp*. Neuronal expression *Ptbx-2::gfp* is marked with arrowheads. Ectopic expression in representative gut nuclei is marked with arrows.

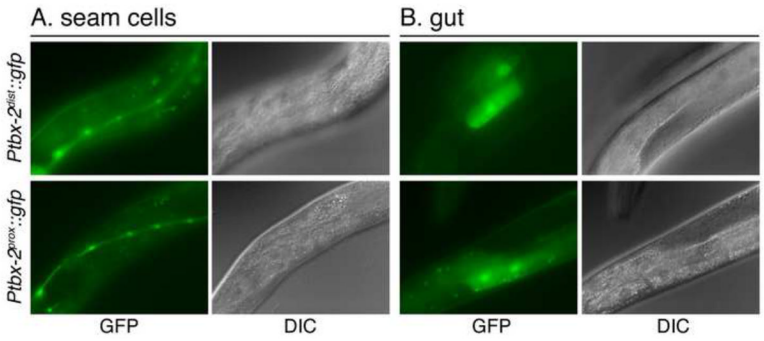

Figure 6. Ectopic *Ptbx-2::gfp* expression in CCAAT-box mutants

Fluorescence (left) and DIC (right) images of L4 or young adult animals expressing *Ptbx-2::gfp* reporters in which the distal or proximal CCAAT-boxes were mutated to AACCT. *Ptbx-2^{prox}::gfp* and *Ptbx-2^{dist}::gfp* are expressed in the seam cells (A) and gut (B) as described in the text.

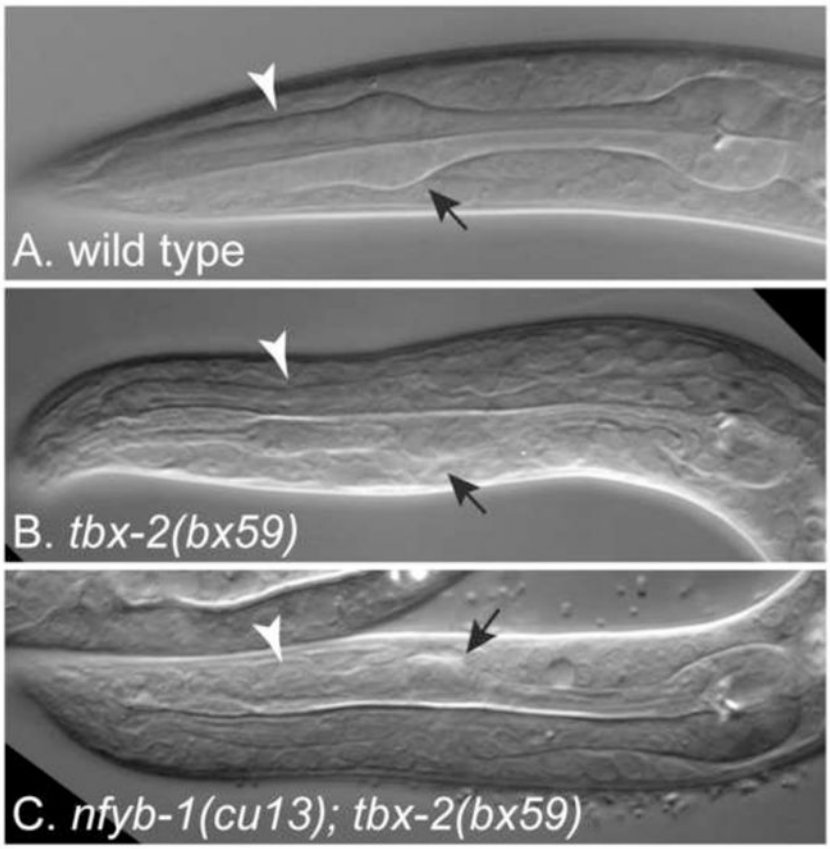

Figure 7. Pharyngeal morphology is similar in *tbx-2(bx59)* and *nfyb-1(cu13); tbx-2(bx59)*

DIC micrographs of the pharynxes of L1 animals: (A) wild type; (B) a moderately affected *tbx-2(bx59)* animal; (C) an *nfyb-1(cu13); tbx-2(bx59)* double mutant. The procorpus (arrowhead) and the metacorpus (arrow) consisting primarily of ABa-derived pharyngeal muscles are marked. These regions are present but morphologically abnormal in *tbx-2(bx59)* and *nfyb-1(cu13); tbx-2(bx59)* mutants.

Table 1

Frequency of *Ptbx-2::gfp* expression is seam cells and gut

genotype	% animals expressing *Ptbx-2::gfp* in seam cells	% animals expressing *Ptbx-2::gfp* in gut	n
cuIs23[Ptbx2::gfp]	0	6	148
cuIs23; nfya-1(ok1174)	46	100	70
cuEx683[Ptbx-2^{prox}::gfp][a]	93	86	105
cuEx684[Ptbx-2^{prox}::gfp][a]	71	52	85
cuEx680[Ptbx-2^{dist}::gfp][a]	9	8	65
cuEx672[Ptbx-2$^{prox+dist}$::gfp][b]	79	38	29
cuIs23; nfyb-1(cu13)	95	100	81

[a]Expression was scored in L3 to young adult hermaphrodites.

[b]Expression was scored in L2 to young adult hermaphrodites.

Table 2

Viability of *tbx-2(bx59)* and *nfyb-1(cu13)* mutants

genotype[a]	% dead embryos	% arrested L1s	% L4/adult	n
+/+	2	0	98	60
tbx-2(bx59)	6	44	50	64
nfyb-1(cu13)	4	0	95	22
nfyb-1(cu13); tbx-2(bx59)	2	98	0	47

[a]Phenotypes were scored in the progeny of L4 hermaphrodites shifted to 25°C. Animals reaching L4 and adult stages were scored after 3 days.

Table 3

Effect of *nfyb-1(RNAi)* on *tbx-2(bx59)* and *nfyb-1(cu13); tbx-2(bx59)* mutants

genotype[a]	% L4/Adult	n
+/+	84	400
tbx-2(bx59)	46	483
nfyb-1(cu13); tbx-2(bx59)[b]	0	422
nfyb-1(RNAi)[b]	92	350
nfyb-1(RNAi); tbx-2(bx59)[b]	11	385
nfyb-1(RNAi); nfyb-1(cu13); tbx-2(bx59)[b]	0	135

[a]Phenotypes were scored in the progeny of L4 hermaphrodites shifted to 25°C. Animals reaching L4 and adult stages were scored after 3 days.

[b]Pooled data from embryos collected 12–16 and 24–27 hr post-injection. No differences were observed at these different time points.

Table 4

Effect of *tbx-2(RNAi)* on *nfyb-1(cu13)* and *nfyb-1(cu13); tbx-2(bx59)* mutants

genotype[a]	%L4/Adult	n
+/+	82	453
nfyb-1(cu13)	90	451
nfyb-1(cu13); tbx-2(bx59)	0	315
tbx-2(RNAi)[b]	31	452
tbx-2(RNAi); nfyb-1(cu13)[b]	7	343
tbx-2(RNAi); nfyb-1(cu13); tbx-2(bx59)[b]	0	289

[a]Phenotypes were scored in the progeny of L4 hermaphrodites shifted to 25°C. Animals reaching L4 and adult stages were scored after 3 days.

[b]Pooled data from embryos collected 12–16 and 24–27 hr post-injection. No differences were observed at these different time points.