Role of T-box gene *tbx-2* for anterior foregut muscle development in *C. elegans*

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Abstract

During organogenesis, pluripotent precursor cells acquire a defined identity such as muscle or nerve. The transition from naïve precursor towards the differentiated state is characterized by sequential waves of gene expression that are determined by regulatory transcription factors. A key question is how transcriptional circuitry dictates the succession of events that accompanies developmental competence, cell fate specification and differentiation. To address this question, we have examined how anterior muscles are established within the *Caenorhabditis elegans* foregut (pharynx). We find that the T-box transcription factor *tbx-2* is essential to form anterior pharyngeal muscles from the ABa blastomere. In the absence of *tbx-2* function, ABa-derived cells initiate development normally: they receive glp-1/Notch signaling cues, activate the T-box gene TBX-38 and express the organ selector gene PHA-4/FoxA. However, these cells subsequently arrest development, extinguish PHA-4 and fail to activate PHA-4 target genes. *tbx-2* mutant cells do not undergo apoptosis and there is no evidence for adoption of an alternative fate. TBX-2 is expressed in ABa descendants and depends on activation by *pha-4* and repression by components of glp-1/Notch signaling. Our analysis suggests that a positive feedback loop between *tbx-2* and *pha-4* is required for ABa-derived precursors to commit to pharyngeal muscle fate.

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Keywords: Tbx2; Tbx3; Tbx4; Tbx5; omb; FoxA; Pharynx; pha-4; Notch; glp-1; tbx-38

Introduction

During the orderly progression of development, multi-potent progenitors adopt specific cell fates. For example, in the vertebrate retina, a pool of retinal precursors produces seven distinct cell types in response to proneural transcription factors and intercellular signaling pathways (Ahmad et al., 2004; Kim et al., 2005). During *Caenorhabditis elegans* embryogenesis, a progenitor population generates the seven cell types of the pharynx (Albertson and Thomson, 1976). Many genes have been identified that are expressed in different pharyngeal cell types, but none is essential to specify a particular cell fate (Ao et al., 2004; Gaudet and Mango, 2002; Okkema and Fire, 1994; Thatcher et al., 2001). Thus, a critical question is how the pharyngeal precursors are patterned to produce the seven cell types of the mature pharynx. Here we explore how one cell type, anterior pharyngeal muscle, is established during embryogenesis.

Studies over the past 10 years have lead to an outline of how the pharyngeal precursors are formed during embryogenesis. At the 4-cell stage, two blastomeres, ABa and EMS, are destined to generate all of the pharyngeal cells, as well as additional, non-pharyngeal cells (Sulston et al., 1983). ABa will give rise to many anterior pharyngeal cells, including anterior muscles. The appropriate development of ABa depends on intercellular signaling by the Notch receptor orthologue GLP-1 at the 12- to 15-cell stage (Hutter and Schnabel, 1994; Mango et al., 1994b; Moskowitz et al., 1994; Priess and Thomson, 1987; Priess et al., 1987). At the 24-cell stage, the ABa descendants express the T-box genes TBX-37 and TBX-38, and these proteins function in combination with GLP-1-dependent signaling to activate the FoxA factor PHA-4 at the 44-cell stage (Good et al., 2004). *pha-4* is a selector gene that specifies pharyngeal identity for both ABa and EMS descendants. In the absence of *pha-4* activity, cells destined to become part of the pharynx are transformed into ectoderm (Horner et al., 1998;
Conversely ectopic **pha-4** is sufficient to drive non-pharyngeal cells towards a pharyngeal fate (Horner et al., 1998). These effects are global, affecting many different cell types within the pharynx. How, then, are particular pharyngeal cell fates established? The transcription factor ceh-22/NKx2.5 is expressed in a subset of pharyngeal muscles and its loss results in feeding abnormalities (Okkema et al., 1997). Nevertheless, pharyngeal muscles are still present in ceh-22 mutants (Okkema and Fire, 1994), indicating that additional factors are required.

Here we identify the T-box factor tbx-2 and demonstrate that it is critical to establish pharyngeal muscles derived from ABA. The T-box family of transcription factors is defined by the T-box DNA-binding domain, and can function as either activators or repressors (Wilson and Conlon, 2002). T-box genes are essential for developmental processes such as patterning the germ layers (Brachyury), vertebrate limb bud development (Tbx4, Tbx5), and cardiac development (Tbx2, Tbx20) (Harrelson et al., 2004; Singh et al., 2005). The most similar vertebrate homologues of C. elegans tbx-2 are H. sapiens Tbx2/3 (pBlast E value 4.4e–8 and TBX4/5 (E value 6.2e–67). Tbx2/3/4/5 represent a subgroup of closely related T-box genes that were likely generated by two duplication events (Agulnik et al., 1996). Tbx2/3 are involved in feeding abnormalities (Okkema et al., 1997). Nevertheless, missexpression of Tbx2 in chick embryos leads to transformation of digit III to IV (Suzuki et al., 2004), whereas in mouse, loss of Tbx2 leads to digit IV duplications (Harrelson et al., 1996). Conversely ectopic

.. _Material and methods:

**Material and methods**

**Nematode strains**

*C. elegans* was Bristol N2, maintained as described (Brenner, 1974). The following strains were used: ceh-22::GFP (Okkema et al., 1997), fog-2(q71) pha-4(q490)/sta-3(q265) V (Mango et al., 1994a), pha-4(q490)/sta-3(q265) Tbx2/3(q160)/

**RNA interference**

DNA templates for F21H11.3 (tbx-2) and C35D10 (pha-4) were obtained in pl.4440 dsT7 RNAi plasmid from the ORFeome library (Reboul et al., 2003), C35D10.9 (pha-4) or the T7 terminator primer 5′-GACATTAGACGATTAGACGATTAGGGA-3′ and 5′- GCATCGTTTACAGCAAGTGGAC-3′ (Gautier and Mello, 2002), 40 ng/μl prf4 and 60 ng/μl herring sperm DNA. avr-15::GFP (Dent et al., 1997) transgenic worms were created by microinjection of 89 ng avr-15::GFP, 39 ng prf4, 62 ng/μl herring sperm DNA and 1.0 ng/μl 1 kb DNA ladder (Gibco/BRL) into wild-type worms. avr-15::GFP was integrated by gamma irradiation (Mello and Fire, 1993).

The 2.5 kb promoter DNA was useed to produce PCR amplification with forward primer 5′-GATTTATTCTCGTGCCTCGGTTCTCCTTTGAAG-3′ and reverse primer 5′-AGCTTGTTCTCTTTTGAATTTCACATTTCATTTTG-3′ (penultimate codon from Y54H5). The product was re-amplified with nested primers 5′-ATTGTATTCCTGGCTCGTGGCTCT-3′ and reverse primer 5′-AGGCTTTGACAACAAATCGA-3′. The PCR product was useed to generate the 1 kb promoter fusion (Alder et al., 2003).

The 5.2 kb promoter DNA was used to produce PCR amplification with forward primer 5′-GATTTATTCTCGTGCCTCGGTTCT-3′ and reverse primer 5′-AGGCTTTGACAACAAATCGA-3′ (penultimate codon from Y54H5). The product was re-amplified with nested primers 5′-TTAAGGTATTCCTGGCTCGTGGCTCT-3′ and 5′-AGTGGCACTGCAGGTCGACT-3′. The PCR product was used to generate the 5.2 kb promoter DNA into pPD95.75 (Fire et al., 1995).

RNAi constructs were transcribed using the AmpliScribe T7 polymerase kit (Epicenter) and linearized with restriction enzyme into EcoRI and SalI. dsRNA was purified with RNAeasy Mini spin columns (Qiagen), and annealed in 50 mM NaCl, 10 mM Tris pH 7.5. 20 ng/μl DNA templates for F21H11.3 (tbx-2) and C35D10.9 (pha-4) were obtained in pl.4440 dsT7 RNAi plasmid from the ORFeome library (Reboul et al., 2003), C35D10.9 (pha-4) or the T7 terminator primer 5′-GACATTAGACGATTAGACGATTAGGGA-3′ and 5′- GCATCGTTTACAGCAAGTGGAC-3′ (Gautier and Mello, 2002), 40 ng/μl prf4 and 60 ng/μl herring sperm DNA. avr-15::GFP (Dent et al., 1997) transgenic worms were created by microinjection of 89 ng avr-15::GFP, 39 ng prf4, 62 ng/μl herring sperm DNA and 1.0 ng/μl 1 kb DNA ladder (Gibco/BRL) into wild-type worms. avr-15::GFP was integrated by gamma irradiation (Mello and Fire, 1993).

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was performed using HT115 bacteria transformed with the *ced-3*, *ced-4*, or *tbx-7*-containing plasmids using standard procedures (Kamath et al., 2003).

**Heat shock**

HS: *tbx-2* was PCR amplified from ORFeome clone 10052@F12 using primers 5′-AAGCTAGCGGATCCCCATGGCATTCAATCATTTGC-3′ and 5′-TTGGTACCGTCGACTTAAGGCTTTGACAACAAATCG-3′ and cloned into Topo TA pCR2.1 (Invitrogen). The resulting plasmid was digested with NheI and KpnI and *tbx-2* moved into pPD49.78 and pPD49.83 (Firelab vectors ftp://www.ciemb.edu/pub/FireLabInfo, Fire et al., 1990). 0.02 ng/μl HS*: *tbx-2* (pPD49.78), 0.02 ng/μl HS*: *tbx-2* (pPD49.83), 0.05 ng/μl *myo-2*: *GFP*: *His2B*, 40 ng/μl pRF4, and 60 ng/μl herring sperm DNA were injected into wild-type worms to obtain stable lines.

Transgenic HS*: *tbx-2*, *myo-2*: *GFP*: *His2B* and control *myo-2*: *GFP*: *His2B* embryos were isolated and treated in 0.2 ml PCR tubes for 30 min at 33°C, then recovered at 20°C. Embryos were examined for reporter gene activity 11.5 h post heat shock. No heat shock controls were incubated at 20°C for 12 h.

**Staining and image analysis**

Staining with 3NB12, anti-intermediate filament, or 9.2.1 antibodies, was performed using standard procedures except slides were incubated in methanol followed by acetone, for 3 min each (modification of Albertson, 1984; modification of Mango et al., 1994a). Larvae were incubated with TNB [100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% blocking reagent] containing 10% normal goat serum (NGS) for 30 min. Slides were incubated 12–18 h at 15°C using primary antibodies diluted in TNB/NGS: 3NB12 (1:5) (Okamoto and Thomson, 1985; Priess and Thomson, 1987), αIF (1:3) (Priess and Thomson, 1987; Pruss et al., 1981), 9.2.1 (1:5000) (Miller et al., 1983), αPHA-4 N-terminal (1:200), αTBX-38 (1:2) (Good et al., 2004), αLIN-26 (1:1000) (Labouesse et al., 1996), and αGFP mAB3580 (1:250) (Chemicon). Slides were washed three times in TBS + 0.5% Tween20, before addition of donkey anti-mouse or anti-rabbit, Cy-2 or Cy-3-conjugated antibodies (1:200 dilution, Jackson ImmunoResearch) for 2 h. Slides were washed 3× in TBS + Tween20 and mounted in 50.0% glycerol, 0.006 M Na Citrate, 0.05 M NaH2PO4, 0.5 mg/ml 4′,6-diamidino-2-phenylindole (DAPI), 25 mg/ml 1,4-diazobicyclo-[2.2.2]-octane (DABCO) and a pinch of p-phenylenediamine.

Confocal microscope images were captured on an Olympus FluoView confocal microscope with FluoView 2.1 software. Z-series were computed by ImageJ software using maximum intensity. PHA-4: *GFP*: histone nuclei were counted using live embryos and a Zeiss axioskop, fitted with a Ludl focus drive (0.4-μm step setting), Roper Scientific Micromax digital camera, Zeiss Attoarc fluorescent burner (5% power setting for time-lapse), and Improvision Openlab 3.1.5 software. 4D movies were acquired using a Applied Precision Delatvision RT with 0.2- to 0.4-μm step sections taken every 15 min. Images were deconvolved using Applied Precision RTView suite software with default settings.

**Results**

**Discovery of the tbx-2 locus**

Our interest in *tbx-2*(F21H11.3) derived from a global RNAi screen of genes with expression enriched within pharyngeal cells (P.A.S., C. Armstrong, M. Vidal and S.E.M., unpublished; Ao et al., 2004). Fig. 1. The *tbx-2* locus and its predicted product. (A) *tbx-2* consists of five exons based on EST alignments, GeneFinder, and ORFeome 3.1 (Lamesch et al., 2004). We constructed a GFP reporter with 5.2 kb of sequence upstream of the ATG and the open reading frame fused to GFP at the predicted carboxyl terminus. This construct uses the *unc-54* 3′UTR and polyadenylation site (Fire et al., 1990). The ok529 deletion spans exons four (red). (B) TBX-2 has sequences similar to the amino-terminal repression domain of vertebrate Tbx2 and Tbx3 (He et al., 1999), the conserved T-box (underlined, Wilson and Conlon, 2002), and nuclear localization signal (orange) (Carlson et al., 2001; Miyahara et al., 2004). The ok529 mutation results in an in-frame deletion of exon 4 (red), compromising the conserved T-box DNA-binding domain. (C) Alignment of the amino terminus of *C. elegans* TBX-2 with sequences from the amino terminal repression domain of vertebrate Tbx2/3 (He et al., 1999).
al., 2004; Gaudet and Mango, 2002). RNAi of \( \text{tbx-2} \) led to defects in the anterior pharynx, an Uncoordinated phenotype and a first larval stage arrest (described below). To confirm the phenotype, we obtained the deletion allele \( \text{ok529} \) from the Gene Knockout Consortium (http://celeganskoconsortium.omrf.org/). The \( \text{tbx-2(ok529)} \) deletion eliminated bases 5,132,217 to 5,133,298 of chromosome III (1082 bp), resulting in the in-frame removal of exon four (Figs. 1A, B). Exon 4 contains part of the DNA-binding domain (Miyahara et al., 2004), which in the homologous T-box factor Brachyury directly contacts DNA (Muller and Hermann, 1997).

Hypothetically, \( \text{tbx-2(ok529)} \) could give rise to a protein with either partial activity or an interfering dominant-negative function. To determine if either of these possibilities was likely to be true, we examined the phenotype of \( \text{tbx-2(ok529)} \) mutants in which \( \text{tbx-2} \) was further inactivated by RNAi. Heterozygous \( \text{tbx-2(ok529)+/} \) mothers were injected with dsRNA and their progeny scored for the terminal phenotype. 100% of progeny from either wild-type or \( \text{tbx-2(ok529)+/} \) mothers arrested as embryos or larvae, indicating that RNAi was effective. For \( \text{tbx-2/+} \) mothers, 27% of their progeny arrested with a pharynx phenotype identical to \( \text{tbx-2(ok529)} \) homozygotes (n=44, Table 1, Fig. 2D), suggesting that reduction of TBX-2 protein by RNAi did not lead to a more severe phenotype for \( \text{tbx-2(ok529)} \) homozygotes. The remaining 73% of progeny arrested with a slightly weaker phenotype that resembled \( \text{tbx-2(RNAi)} \) of wild-type worms (Fig. 2B). These animals were likely of the genotype \( \text{tbx-2/+} \) and +/+ . We conclude \( \text{tbx-2(ok529)} \) is a strong loss-of-function, possibly null, allele. This is in agreement with data from Roy Chowdhuri et al. examining this allele and a second, null allele (S. Roy Chowdhuri and P. Okkema, personal communication).

Many \( \text{C. elegans} \) T-box factors function in pairs, including \( \text{tbx-3} \) and \( \text{tbx-8} \) (Andachi, 2004; Pocock et al., 2004). \( \text{tbx-7} \) has the greatest sequence similarity to \( \text{tbx-2} \) (62% similar, 2e−55), and is located 0.63 map units from \( \text{tbx-2} \), suggesting the two share a common ancestor gene. We investigated if loss of \( \text{tbx-7} \) (ZK328.8) would enhance the pharynx phenotype associated with \( \text{tbx-2} \). Neither the \( \text{tbx-7} \) allele \( \text{tm478} \) nor \( \text{tbx-7(RNAi)} \) resulted in a phenotype when examined alone. Nor did we observe a stronger phenotype associated with \( \text{tbx-2(ok529)} \) \( \text{tbx-7(RNAi)} \) double inactivation compared to \( \text{tbx-2(ok529)} \) single mutations, as monitored under the light microscope (\( n=61 \), Table 1). These data, as well as the lack of a \( \text{tbx-7} \) homolog in \( \text{C. briggsae} \), suggest \( \text{tbx-7} \) does not contribute significantly to pharynx development, and therefore we focus on \( \text{tbx-2} \) alone.

\( \text{tbx-2} \) is required for anterior pharynx muscle development

The most striking phenotype associated with \( \text{tbx-2} \) mutations was a loss of anterior pharyngeal muscles. Wild-type \( \text{C. elegans} \) possesses eight rings of pharyngeal muscles (Fig. 2A, Table 2) (Albertson and Thomson, 1976). \( \text{tbx-2} \) mutants lacked expression of muscle markers \( \text{3NB12} \) (Okamoto and Thomson, 1985; Priess and Thomson, 1987) and 9.2.1 (Miller et al., 1983) in 2/3 pm3 cells, 1/3 pm4 cells, and 1/3 pm5 cells (Table 2, Figs. 2E–H). By contrast, posterior muscles pm6, pm7 and pm8 looked wild type, indicating that these cells were not affected. CEH-22-:GFP expression showed a similar pattern, with one pm3, two pm4, and one pm5 cell expressing the reporter (Figs. 2I, J). These numbers suggested that muscles derived from ABa were altered or missing, whereas those from EMS were not (compare Tables 2 and 3). Three additional markers supported this idea. First, we counted pharyngeal muscle nuclei with the nuclear muscle marker \( \text{myo-2} \) :GFP (Miller et al., 1986). Wild-type embryos had an average of 35.5 GFP+ nuclei (\( n=9 \) embryos), close to the published number of pharyngeal nuclei (37, 39).
Albertson and Thomson, 1976). By contrast, tbx-2 mutants had an average of 19 GFP+ nuclei, all of which were located in the posterior of the head ($n = 7$). This number agreed well with the number of pharyngeal muscles produced by EMS in wild-type embryos (18, Albertson and Thomson, 1976), consistent with an absence of ABa-derived muscles. Second, we examined ITR-1::GFP, which is normally expressed in nine muscle cells of the central pharynx (pm4, pm5, pm6, and weakly in pm3; Dal Santo...
et al., 1999). In tbx-2 mutants, ITR-1∷GFP was detected in three cells with pm6-like characteristics, based on location and morphology, but rarely in any other pharynx muscle cell (Figs. 2K, L; \( n = 7 \) mutant vs. \( n = 9 \) wild-type larvae). Mutant animals also expressed ITR-1∷GFP in other cell types such as midgut and pharyngeal valves, confirming the presence of the extrachromosomal array. Third, we saw a reduction in the anterior expression of AVR-15∷GFP, which is normally expressed in pm4/pm5 and extra-pharyngeal neurons (Figs. 2M–N; Dent et al., 1997). We conclude that tbx-2 activity is required for anterior muscle development, specifically those muscles generated by the ABa blastomere.

We examined whether other ABa-derived pharyngeal cells were affected by tbx-2 mutations. ABa normally produces 7/9 pharyngeal marginal cells (Albertson and Thomson, 1976; Sulston et al., 1983), which express pax-1∷GFP early in development and intermediate filaments late (Bartnik et al., 1986; Portereiko et al., 2004). In tbx-2 mutants, PAX-1∷GFP was expressed in eight nuclei, like the wild type (\( n = 12 \)), and intermediate filament staining appeared normal (Figs. 2O, P).

Thus, marginal cells are made in tbx-2 mutant embryos, and tbx-2 is not required for all ABa-derived pharyngeal cells.

### The fate of anterior pharyngeal muscles in mutants

When does tbx-2 affect anterior muscle development? To address this question, we examined early markers of anterior pharyngeal muscle development. We also tested for possible alternative fates in mutant embryos. This analysis suggested that pharyngeal cells initiate muscle development but then arrest or de-differentiate in the absence of tbx-2 activity.

We surveyed markers of anterior pharyngeal muscles to determine the earliest defects associated with tbx-2 mutations. First we examined TBX-38, which is normally activated at the 24-cell stage in ABa descendants (Good et al., 2004). All embryos from tbx-2/+ mothers expressed TBX-38 between the 16-cell stage and the 100-cell stage (\( n = 17 \), Figs. 3A, B). Second, we examined PHA-4 using an integrated pha-4∷GFP reporter (Alder et al., 2003). This reporter normally expresses GFP beginning at the 28-cell stage and continuing throughout the life of the worm (Alder et al., 2003; Horner et al., 1998). Activation of PHA-4∷GFP was normal in EMS descendants at the 28-cell stage and in ABa descendants at the 44-cell stage in mutant embryos (Table 4). This result suggested that glp-1-mediated signaling and tbx-37 and/or tbx-38 were functional in tbx-2 mutants, since both signaling and TBX activity are required for PHA-4 activation in ABa descendants (Good et al., 2004; Kalb et al., 1998; Mango et al., 1994a). We conclude that the earliest stages of ABa development are normal in tbx-2 mutants.

Next we analyzed markers of later development and differentiation. There was an average of 13 fewer PHA-4∷GFP+ cells at the comma stage of embryogenesis compared to the wild type, 16 fewer at the 1.5- to 2-fold stage and 18 fewer by the L1 stage (Figs. 3C–F, Table 4). Staining for endogenous PHA-4 confirmed this dramatic decrease in mutant animals (data not shown). These data were corroborated by examining the PHA-4 target gene ceh-22, which is normally activated in pm3–pm5 and pm7 at the pre-bean stage (Okkema and Fire, 1994). We observed 4–6 CEH-22∷GFP+ cells at the pre-bean stage in both mutant and wild-type embryos (Table 5). Subsequently, the number of CEH-22∷GFP+ cells increased to 18 in wild-type embryos, but only to 13 cells in mutant animals (Figs. 3G–J, Fig. 4, Table 5). The expressing cells appeared to be part of pm3, pm4, pm5 and all of pm7, based on position and morphology.

### Table 2

Pharynx muscle nuclei and cell fusion

<table>
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<tr>
<th>Pharynx muscle nuclei</th>
<th>Embryonic cell lineage</th>
<th>Mature pharynx cell</th>
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</tr>
<tr>
<td>pm1DR</td>
<td>AB.araaappp</td>
<td>pm1</td>
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ABa lineage pharynx muscle cells are shown in bold type.

### Table 3

tbx-2 is required to produce pharyngeal muscles

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<td>3</td>
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<td>3NB12</td>
<td>WT</td>
<td>3</td>
<td>3</td>
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<td>tbx-2(ok529)</td>
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9.2.1 recognizes pharyngeal myosin (Miller et al., 1983); 3NB12 recognizes pharyngeal muscle (Okamoto and Thomson, 1985; Priess and Thomson, 1987). Cell designations based on position and morphology.
Normally, ABa generates 19 embryonic pharyngeal muscle cells, of which seven belong to pm3–5; the remaining twelve being the pm1 and pm2 cells (Table 2) (Sulston et al., 1983). Thus, the number and location of lost CEH-22∷GFP+ cells support the notion that tbx-2 affects ABa-derived muscles (Figs. 2I, J). We suggest that progression of muscle development is aborted in tbx-2 mutants since they cannot maintain PHA-4 expression and fail to activate CEH-22.

We considered three developmental options for anterior muscles in tbx-2 mutants. First, they could undergo apoptosis; second, they could adopt an alternative cell fate; third they could remain as undifferentiated or de-differentiated pharyngeal precursors. To explore apoptosis, we inactivated the cell death pathway using ced-3(RNAi) or ced-4(RNAi) (Joshi and Eisenmann, 2004; Yuan and Horvitz, 1990). We looked at the number of arrested tbx-2(ok529) larvae after ced-3 or ced-4 was inactivated by RNAi and found it identical to control (Table 1). Moreover, ten mutant larvae fed ced-3 dsRNA were phenotypically indistinguishable from non-RNAi controls (data not shown), nor did we observe extra cell corpses in tbx-2 homozygous embryos. The effectiveness of RNAi for ced-3 or ced-4 was examined in the cell corpse engulfment mutant ced-10(n1993) (Kinchen et al., 2005). We found ced-3(RNAi) or ced-4(RNAi) essentially eliminated cell corpses in terminal ced-10(n1993) embryos, demonstrating apoptosis had been reduced by RNAi (data not shown). From these data, we conclude that tbx-2 mutant cells do not undergo extra apoptosis.

Next we tested whether the pharyngeal muscles adopted an alternative fate in the absence of tbx-2. In embryos bearing null alleles of pha-4, pharyngeal cells are converted into ectoderm, which expresses LIN-26 (Horner et al., 1998; Labouesse et al., 1996). LIN-26 expression was examined in 26 tbx-2(ok529) homozygotes, confirmed by the morphology of the anterior pharynx; none activated ectopic LIN-26 or AJM-1 in the anterior pharyngeal region (Figs. 5A–D). We conclude that anterior muscles do not undergo a cell fate transformation to

![Fig. 3](image-url)

Fig. 3. tbx-2 is required for progression of pharyngeal muscle development. (A, B) TBX-38 (red) is expressed normally in all embryos from tbx-2(ok529)/+ mothers (DAPI, blue). (C) Wild-type comma-stage embryo with 94 PHA-4∷GFP+ cells. (D) A tbx-2(ok529) comma-stage embryo with 93 PHA-4∷GFP+ cells. (E) Embryo C viewed 30 min later with 94 PHA-4∷GFP+ cells while embryo D had only 81 PHA-4∷GFP+ cells (F). (G) WT and (H) tbx-2(ok529) embryos had approximately the same number of ceh-22∷GFP expressing cells at the bean stage (arrows), when CEH-22∷GFP is first detectable. Following the same embryos in panels G and H until the two-fold stage, wild-type embryos consistently have more ceh-22∷GFP expressing cells (I, arrows) compared to tbx-2(ok529) embryos (J, arrows). (K) tbx-2∷GFP WT 8E embryo and (L, arrows) tbx-2∷GFP pha-4(RNAi) embryo with TBX-2∷GFP expressing cells. (M) Differential interference contrast micrograph (DIC) of a pha-4(q490) larva lacking a pharynx and (N) the same larva showing no TBX-2∷GFP in the head other than in two non-pharyngeal cells (arrows). Each embryo is ~ 50 μm long.
ectoderm in \textit{tbx-2} mutants. We also examined the pharynges of \textit{tbx-2} embryos and larvae for markers of intestinal cells, pharyngeal glands (J126), body-wall muscles (MYO-3) and neurons (UNC-86). Wild-type staining was observed for each of these (data not shown). These data suggest that ABA-derived muscle precursors do not adopt a new fate or undergo apoptosis in \textit{tbx-2} mutants. We favor the notion that these cells arrest their development or de-differentiate.

**TBX-2:\textsuperscript{::}GFP is expressed in pharyngeal muscles**

Given the effects on anterior muscle development, we wished to determine when and where TBX-2 was expressed. We relied on a \textit{tbx-2:\textsuperscript{::}GFP} translational fusion that included 5.2 kb upstream sequences and positioned GFP at the carboxyl terminus (Fig. 1A). We were unable to obtain TBX-2 antibodies, either from published sources or our own production, however, our \textit{tbx-2:\textsuperscript{::}GFP} reporter result agrees well with data from a global transcription profile that detected \textit{tbx-2} transcripts starting at the 4E stage (~102 cell stage, 143 min after the 4-cell stage (Baugh et al., 2003)), and with mid-gastrula expression observed by in situ hybridization (Shin-i and Kohara, \url{http://nematode.lab.nig.ac.jp}).

The uncoordinated phenotype was rescued for all \textit{tbx-2(ok529)} worms in transgenic strains, and the pharynx developmental phenotype was sufficiently rescued to allow embryos to hatch and initiate larval development. However, mutant larvae carrying either \textit{tbx-2} or \textit{tbx-2:\textsuperscript{::}GFP} failed to reach adulthood (e.g., 4/72 F1 \textit{tbx-2:\textsuperscript{::}GFP} progeny examined). The pharynx appeared fully formed in arrested \textit{tbx-2(ok529)}; \textit{tbx-2\textsuperscript{::}GFP} larvae, and \textit{tbx-2\textsuperscript{::}GFP} expression was present in the both MS- and ABA-derived pharynx cells. These data indicate that the \textit{tbx-2\textsuperscript{::}GFP} reporter contains sequences appropriate for expression within ABA-derived pharyngeal cells, but may lack sequences required for larval growth.

**Regulation of \textit{tbx-2} expression**

How is \textit{tbx-2} expression regulated? We generated translational GFP reporters containing 1.0, 1.2, 2.3, 2.4, 3.2, 4.1, 4.7, and 5.2 kb of putative promoter DNA to identify the minimal upstream sequences necessary for regulation (Fig. 7A). Only the 5.2 kb promoter construct conferred expression at the 8E stage, indicating that sequences between 4.7 and 5.2 kb were necessary to initiate expression appropriately (Figs. 7B–E). All \textit{tbx-2\textsuperscript{::}GFP} reporter arrays between 4.7 and 2.3 kb were similar, with expression initiating at the comma stage, but
weakly and in fewer cells than the 5.2 kb reporter (Figs. 7C, G, K). For the shortest promoters of 1.0 kb and 1.2 kb, we observed TBX-2∷GFP only in presumed pharyngeal neurons (Figs. 7D, H, L).

We removed tbx-2 intronic sequences by generating a 5.2 kb transcriptional reporter that fused GFP to the ATG start site within the first exon (tbx-2p∷GFP). GFP was activated at the 100-cell stage, like the translational reporter, but expression soon spread to additional, extra-pharyngeal cells and persisted throughout development (Figs. 7E, I, M). These data indicate that there are likely to be negative regulatory elements within one or more tbx-2 introns, which confine expression to the pharynx and body wall muscles. These data suggest that tbx-2 regulatory sequences are widely distributed, with both positive and negative cis-regulatory sequences that function at different developmental stages and in different cellular contexts.

Analysis of the sequences between the start codon and 5.2 kb upstream revealed two potential LAG-1 consensus sequences 2383 and 4862 bp upstream of the tbx-2 translation start (RTGGGAA), and thirty bHLH consensus sequences (CANNTG), which could be bound by members of the REF-1 family (Fig. 7A; Neves and Priess, 2005). Five additional bHLH-binding sites are present in the first intron (Fig 7). The REF-1 proteins constitute a family of transcriptional repressors that are activated by Notch signaling (Neves and Priess, 2005). Based on these sequence motifs, we examined if tbx-2 was regulated positively or negatively by the Notch pathway.

TBX-2∷GFP was activated normally in embryos lacking either glp-1 or lag-1 (Fig. 8A). Expression was initially observed in 8.4 cells in lag-1(RNAi) embryos (n=10/10) and in 8.4 cells in glp-1(RNAi) embryos at the 8E stage (n=10/10). This result complements our tbx-2∷GFP reporter analysis, in which constructs with one or no putative LAG-1-binding site activated expression identically (2.3 kb vs. 2.4 kb of upstream sequence; data not shown). These data reveal that glp-1 signaling is not required to activate tbx-2 expression.

After the 8E stage, we observed extra TBX-2∷GFP in embryos lacking lag-1. These cells were located outside of the pharynx. Similarly, we observed de-repression of TBX-2∷GFP when the REF-1 family of repressors were inactivated by RNAi, and this was observed already at the 8E stage (Fig. 8). 15.3 cells expressed the transgene at the 8E stage when REF-1 factors ref-1, hlh-25, hlh-26, hlh-28, and hlh-29 were simultaneously inactivated instead of the usual 8 cells (n=13) (Neves and Priess, 2005). As embryogenesis progressed, additional TBX-2∷GFP+ cells were observed (Fig. 8). Thus, lag-1 is required to repress tbx-2 expression, possibly by activating the REF-1 family of repressors.

Previous experiments have shown that REF-1 factors repress the T-box genes TBX-37 and TBX-38 in many cells at the 28 cell stage (Good et al., 2004). Therefore, it was possible that the activation of TBX-2∷GFP in embryos lacking lag-1 or REF-1 proteins reflected activation by TBX-37 or TBX-38 rather than direct repression by REF-1 proteins. Unfortunately, it was impossible to test this idea since we could not generate a tbx-37 tbx-38 double mutant strain that carried the TBX-2∷GFP reporter stably. We did observe transient TBX-2∷GFP in the posterior pharynx of homozygous tbx-37/38 larvae. Thus, while we cannot distinguish direct vs. indirect repression effects of REF-1 proteins, our data demonstrate that Notch signaling restricts TBX-2 expression to a defined set of cells and is not required to activate TBX-2 within the ABa lineage.
tbx-2 and pha-4 synergize to promote pharyngeal muscle fate

Our findings suggest that TBX-2 functions in combination with PHA-4, and that a positive feedback loop between these factors is required for commitment to pharynx muscle fate. As a first test of this idea, we examined TBX-2::GFP expression in *pha-4* mutant embryos. Strikingly, TBX-2::GFP was activated in 6.9 cells in *pha-4*(RNAi) embryos (*n* = 14), but expression was extinguished by the L1 stage (Figs. 3K–N). As expected, TBX-2::GFP expression was unaffected in body wall muscles and non-pharyngeal cells of head. Thus, *pha-4* and *tbx-2* are each required to maintain expression of the other. As a second test, we reasoned that if pharyngeal muscle development relies on a combination of *tbx-2* and *pha-4*, then exogenous expression of *tbx-2* in the absence of *pha-4* might be sufficient to induce cells to adopt a pharyngeal muscle fate. To investigate this idea, we expressed *tbx-2* ubiquitously in early embryos (≤ 8E) under control of the heat shock promoter (Jones et al., 1986). We observed an average of 40.3 *myo-2*::GFP+ pharyngeal muscles after heat treatment (*n* = 7), compared to 35.7 in wild-type embryos (*n* = 11). These *myo-2*::GFP+ cells were located within the pharynges of L1 larvae, as expected if TBX-2 synergizes with PHA-4, typically in the anterior pharynx and VPI region (data not shown). No excess MYO-2::GFP expressing cells were seen in worms lacking the HS::*tbx-2* construct or in transgenic worms incubated at 20°C. We conclude *tbx-2* can induce cells to adopt a pharyngeal muscle fate but only in the context of PHA-4+ pharyngeal cells. We favor this explanation rather than the
alternative, that tbx-2 promotes division of anterior pharyngeal muscles, since the ectopic cells were the same size as non-heat-shocked pharyngeal muscles and had not become smaller by extra rounds of cell division. It is likely that additional factors are involved in these cell fate decisions since not all PHA-4+ cells adopted a pharyngeal muscle fate, despite widespread TBX-2.

Discussion

Our analysis has made three contributions towards understanding the circuitry that governs cell fate specification in the C. elegans foregut. First, we identified tbx-2, which is required for ABA-derived precursors to adopt a pharyngeal muscle fate (Fig. 9). This is the first locus identified that is essential to produce a particular cell type within the pharynx. Second, tbx-2 is repressed by Notch signaling and the REF family of transcription factors. This regulation confines TBX-2 to ABA descendants, which may be important for limiting the number of pharyngeal muscles. Third, TBX-2 and PHA-4 are mutually dependent on each other to maintain expression, suggesting a direct or indirect positive regulatory loop. We propose that this regulatory loop is essential for commitment to pharyngeal muscle fate since pharyngeal muscles are lost in either pha-4 or tbx-2 mutant embryos.

PHA-4 expression normally initiates at the 2–4E stage whereas TBX-2 initiates at the 8E stage. These time-points correspond precisely to the period when embryonic blastomeres lose their developmental plasticity (Fukushige and Krause, 2005; Gilleard and McGhee, 2001; Horner et al., 1998; Zhu et al., 1998). After this stage, blastomeres are not able to transform to a different cell type identity (reviewed in Labouesse and Mango, 1999). Conversely, the loss of pha-4 or elt-1 eliminates pharynx or epidermis, respectively, demonstrating that these factors are necessary for early patterning (Mango et al., 1994a; Page et al., 1997). An intriguing notion is that the positive regulatory loop between PHA-4 and TBX-2 contributes to the cell fate restriction that occurs in ABA descendants by the 8E....
stage. This model could explain the lack of a transformation in MS-derived cells that express TBX-2. TBX-2 expression initiates after the 8E stage in MS descendants, which may be too late to contribute to cell fate commitment.

pha-4 directly regulates most pharyngeally expressed genes via a TRRTKRY consensus sequence (Gaudet and Mango, 2002). The *C. elegans* *tbx-2* 5.2 kb promoter includes 19 PHA-4 consensus sites, six of which are located within the 4.7–5.2 kb upstream sequence. While not conserved in position, PHA-4-binding sites are also found in *tbx-2* from *C. ramanei* (13) and *C. briggsae* (8). Thus, PHA-4 may activate *tbx-2* transcription directly during the maintenance phase. We note that the large number of consensus sites and the lack of direct alignment of sites complicate the analysis of direct vs. indirect regulation.

Previous studies of the Tbx2/3/4/5 subfamily suggested a role in anteroposterior patterning. For example, misexpression of Tbx2 in the chick limb can convert digit III into digit IV, and Tbx3 can convert digit II to digit III (Suzuki et al., 2004). Similarly, Tbx4 is required for hindlimb development whereas Tbx5 is required for the forelimb bud (Rodriguez-Esteban et al., 1999). These activities, in addition to the contribution of *C. elegans* *tbx-2* for anterior pharyngeal muscles, suggest that an ancient role for the Tbx2/3/4/5 subfamily involves anterior/posterior patterning. However, the phenotypes associated with these genes are not entirely clear cut. For example, inactivation

**Fig. 8.** *ttx-2::GFP* expression is repressed by Notch signaling. (A) *ttx-2::GFP* 5.2 kb translational reporter is de-repressed at the 8E stage when the *ref-1* family is inactivated by feeding RNAi, but activated normally with *glp-1* and *lag-1* inactivation. *Tbx-2::GFP* expression in 8E cell embryos (B, D, F, H, J) and 16E cell embryos (C, E, G, I, K). WT embryos (B, C), compared to feeding RNAi of *glp-1* (D, E), *lag-1* (F, G), and *ref-1* members (H, I) shows de-repression of *ttx-2::GFP* at the 16E cell stage for *lag-1* and *ref-1* members. *pha-4(RNAi)* reduces the number of *Tbx-2::GFP* expressing cells (J, K). Error bars indicate SD. *P* values of *ttx-2::GFP* cell counts of RNAi embryos compared to wild type are *glp-1*, *p* = 0.32; *ref-1 proteins*, *p* = 0.001; *lag-1*, *p* = 0.0003; *pha-4*, *p* = 0.0000003.

**Fig. 9.** A model of pharyngeal muscle specification in the ABa lineage. Notch signaling and *lag-1* are required to activate the organ selector gene *pha-4* in cells destined to become pharynx. *lag-1* also activates the *ref-1* family of transcriptional repressors, and these limit *ttx-2* expression to ABa descendants at ≥8E stage, directly or indirectly. TBX-2 and PHA-4 enter a positive feedback loop, which is required for commitment and developmental progression of anterior pharyngeal muscles by the 1.5-fold/comma stage.
of Tbx5 in mouse leads to loss of FGF expression and forelimb outgrowth, but patterning of the limb field appears normal (Takeuchi et al., 2003). Therefore, the effects of Tbx5 on anterior cells may reflect proliferation rather than anterior vs. posterior fate. In C. elegans, we do not observe alterations in cell division of ABa descendants in tbx-2 mutants. Moreover, FGF, which is an important Tbx target for growth and morphogenesis in vertebrates, is probably not downstream of tbx-2 in C. elegans. FGF ligands and receptors have no described phenotypes in the anterior pharynx (Birnbaum et al., 2005). Rather, our phenotypes are best explained as alterations of cell fate progression.

When lag-1/su(H) or the REF-1 proteins were inactivated, we observe extra cells expressing TBX-2::GFP. These data suggest that LAG-1 and REF-1 members repress tbx-2, to ensure that only some cells progress towards anterior muscle fate. Studies with Drosophila have suggested that one role for Notch is to block the progression of development (Cagan and Ready, 1989). Inhibition of tbx-2 by Notch-signaling components could serve this purpose, by repressing a gene required for developmental progression.

How does TBX-2 function? In other organisms, members of the Tbx2/3/4/5 subfamily of T-box factors can be either activators (e.g., Tbx4, Tbx5) (Takeuchi et al., 2003; Zaragoza et al., 2004) or repressors (e.g., Tbx2, Tbx3) (Carreira et al., 1998; He et al., 1999). Examination of C. elegans tbx-2 reveals that it has sequences similar to the amino terminal repression domain of vertebrate Tbx2 and Tbx3 (Fig. 1C) (He et al., 1999). On the other hand, the carboxyl terminal sequence of TBX-2 is rich in serine, proline and glutamate, suggesting it could function as an activation domain, similar to some other transcription factors (Matsuzaki et al., 1995; Nagy et al., 2002; Stepchenko and Nirenberg, 2004). Thus, a speculative possibility is that in C. elegans, the sole Tbx2 family member functions as both an activator and a repressor, whereas in vertebrates, with four Tbx2 members, activation and repression activities are segregated into different proteins.

TBX-2 is both nuclear and cytoplasmic

We observe TBX-2 in the cytoplasm beginning at the 1.5-fold stage. This location agrees with previous studies where endogenous TBX-2 was observed in the cytoplasm of post-embryonic neurons (Miyahara et al., 2004). Tbx factors from other organisms also accumulate in the cytoplasm, suggesting this may be a conserved mechanism to regulate Tbx proteins (Bruce et al., 2003; Fuchikami et al., 2002; Krause et al., 2004). Our cytoplasmic expression appears filamentous, as though TBX-2 were associated with the cytoskeleton. The pattern resembles that of microtubules, although this idea remains to be tested. The cytoplasmic expression pattern was detected in 12/12 independent transgenic lines, and the cytoplasmic transition was consistent even in the weakest expressing lines; arguing that this is not an over-expression phenotype. Recent studies with chicken Tbx5 suggested this protein could bind to LMP-4 in vitro, and was targeted to actin filaments in COS cells (Krause et al., 2004). Thus, members of the Tbx2 subfamily may be regulated by cytoskeletal association.

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