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# The NK-2 class homeodomain factor CEH-51 and the T-box factor TBX-35 have overlapping function in *C. elegans* mesoderm development

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The *C. elegans* MS blastomere, born at the 7-cell stage of embryogenesis, generates primarily mesodermal cell types, including pharynx cells, body muscles and coelomocytes. A presumptive null mutation in the T-box factor gene *tbx-35*, a target of the MED-1 and MED-2 divergent GATA factors, was previously found to result in a profound decrease in the production of MS-derived tissues, although the *tbx-35(–)* embryonic arrest phenotype was variable. We report here that the NK-2 class homeobox gene *ceh-51* is a direct target of TBX-35 and at least one other factor, and that CEH-51 and TBX-35 share functions. Embryos homozygous for a *ceh-51* null mutation arrest as larvae with pharynx and muscle defects, although these tissues appear to be specified correctly. Loss of *tbx-35* and *ceh-51* together results in a synergistic phenotype resembling loss of *med-1* and *med-2*. Overexpression of *ceh-51* causes embryonic arrest and generation of ectopic body muscle and coelomocytes. Our data show that TBX-35 and CEH-51 have overlapping function in MS lineage development. As T-box regulators and NK-2 homeodomain factors are both important for heart development in *Drosophila* and vertebrates, our results suggest that these regulators function in a similar manner in *C. elegans* to specify a major precursor of mesoderm.

**KEY WORDS: Mesoderm, *C. elegans*, *tbx-35*, *ceh-51*, Tissue specification**

## INTRODUCTION

During metazoan development, embryonic cells must select from among multiple possible fates, and, ultimately, their descendants will produce gene products typical of a differentiated tissue. In the nematode *C. elegans*, early embryonic cells acquire transient, distinct identities after the zygote undergoes a series of asymmetrical cleavages. These form the six so-called 'founder cells', each of which undergoes a stereotyped pattern of cell divisions to give rise to a nearly invariant set of descendants (Fig. 1A) (Sulston et al., 1983). The emergent paradigm of blastomere/lineage specification is that maternal factors first specify blastomere identity by zygotic activation of blastomere-specific factors, which ultimately leads to activation of tissue-specific gene networks (Labouesse and Mango, 1999; Lei et al., 2009; Maduro, 2009). Blastomere-specific factors are transiently expressed and act for a short time in development, whereas tissue-specific factors tend to maintain their expression throughout the lifespan. An understanding of how lineage-specific activation of tissue factors is achieved will close the gap between studies of blastomere fate and studies of tissue identity, generating a comprehensive gene network that describes development.

The 7-cell stage MS blastomere generates many mesodermal cell types, including cells of the pharynx and body musculature (Fig. 1A,C). The gene cascade that specifies MS has been studied for almost two decades (Fig. 1B). Initial specification of MS requires maternal activity of the bZIP/homeodomain factor SKN-1 (Bowerman et al., 1993; Bowerman et al., 1992). Loss of *skn-1* leads to a lack of MS-derived tissues and a somewhat less penetrant loss of endoderm from E, the sister cell of MS (Bowerman et al., 1992). *skn-1* mutants also lack the AB-derived portion of the pharynx owing to failure of a Notch/GLP-1-mediated induction from MS to the AB lineage (Priess et al., 1987; Shelton and Bowerman, 1996). In *skn-1* mutants, mis-specified MS and E cells adopt the fate of the mesectodermal precursor C (Bowerman et al., 1992). In EMS (the mother of MS and E), SKN-1 activates the zygotic *med-1 med-2* (*med-1,2*) divergent GATA factor gene pair (Coroian et al., 2005; Maduro et al., 2001). Loss of *med-1,2* has a similar effect on MS specification as loss of *skn-1*, but a much weaker effect on E specification owing to parallel contributions to endoderm from SKN-1 and other factors (Goszczynski and McGhee, 2005; Maduro et al., 2005a; Maduro et al., 2001). In MS, MED-1,2 activate the T-box factor gene *tbx-35* (Broitman-Maduro et al., 2006). Loss of *tbx-35* has variable effects on MS lineage development and morphogenesis, although the most severely affected mutants resemble *skn-1* or *med-1,2* embryos and lack most tissues made by MS (Broitman-Maduro et al., 2006).

The regulatory cascade initiated by SKN-1 works combinatorially with other factors that restrict MS fate to the appropriate blastomere. Within the EMS lineage, SKN-1 and its target genes collaborate with the Wnt/β-catenin asymmetry pathway to distinguish MS and E identity (Maduro et al., 2002; Rocheleau et al., 1997; Shetty et al., 2005; Thorpe et al., 1997). EMS receives an induction from its posterior sister $P_2$ that ultimately results in differential nucleocytoplasmic localization of the nuclear effector TCF/POP-1 within MS and E, referred to as POP-1 asymmetry (Goldstein, 1992; Lin et al., 1998; Lo et al., 2004; Maduro et al., 2005a; Rocheleau et al., 1999). Within the E cell, reduced nuclear POP-1 permits POP-1 to function as an

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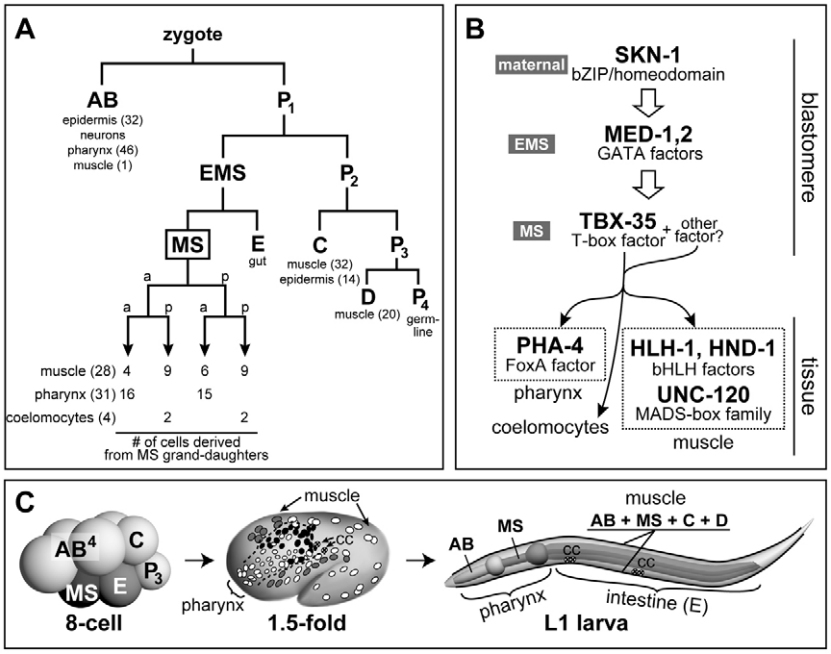



**Fig. 1. Developmental context of the MS lineage and its gene regulatory network.** (**A**) Partial cell lineage showing the production of major tissue types (number of cells in brackets) from early blastomeres (Sulston et al., 1983). The MS lineage is expanded to show the origin of pharynx, muscle and coelomocytes. (**B**) Gene regulatory network for MS specification [modified with permission from Maduro (Maduro, 2009)]. (**C**) Embryo stages. Blastomeres are indicated on the 8-cell stage embryo. In the 1.5-fold embryo, all pharynx nuclei, and body muscle nuclei of the left half of the embryo, are shown. Darker-shaded nuclei are those derived from MS. The left-side embryonic coelomocytes (cc) are shown as circles with an X. For the L1 larva, tissues are indicated along with their blastomere of origin. A *C. elegans* embryo is ~50 μm long. Here and in subsequent figures, anterior is to the left and dorsal is up.

endoderm activator through association with the divergent β-catenin SYS-1 (Huang et al., 2007; Phillips et al., 2007). Blockage of the induction, or of the components that act upstream of POP-1, results in EMS dividing to produce two MS-like cells (Goldstein, 1992; Rocheleau et al., 1997; Rocheleau et al., 1999; Thorpe et al., 1997). Outside of the EMS lineage, multiple factors block inappropriate expression of SKN-1 or prevent its timely degradation, either of which can otherwise lead to ectopic mis-specification of MS or E fates (Lin, 2003; Mello et al., 1992; Page et al., 2007; Shirayama et al., 2006).

The organ-identity factors that specify the two major tissues made by MS, pharynx and muscle, have been well characterized. Pharynx is specified by FoxA/PHA-4 (Horner et al., 1998; Kalb et al., 1998), which is at the top of a network of at least several hundred genes (Gaudet and Mango, 2002) that includes the pharynx muscle-specific gene *ceh-22* (Okkema and Fire, 1994). Body muscle is specified by the activity of three regulators, MyoD/HLH-1, HAND/HND-1 and SRF/UNC-120 (Fukushige et al., 2006). All three genes have overlapping function, as each can specify muscle fate when overexpressed and muscle specification is blocked only when the activity of all three has been compromised (Fukushige et al., 2006). Approximately 1300 genes are known to be enriched for expression in muscle (Fox et al., 2007), suggesting that HLH-1, HND-1 and UNC-120 are at the top of a complex tissue-specific muscle gene network.

In the present study we identify the NK-2 homeobox gene *ceh-51* as a direct target of TBX-35, and present evidence that CEH-51 and TBX-35 have distinct and shared functions. Whereas loss of *ceh-51* function causes subtle muscle and pharynx defects and larval lethality, simultaneous loss of *ceh-51* and *tbx-35* results in a highly penetrant loss of MS-derived tissues and an embryonic arrest phenotype that is strikingly similar to that of *med-1,2(–)* embryos, thus explaining the weaker phenotype of single *tbx-35* mutants. Our results add an important regulator, CEH-51, to the MS gene regulatory network, and suggest that combinatorial control of mesoderm through T-box and NK-2 factors has been evolutionarily conserved.

## MATERIALS AND METHODS

### Strains used

*C. elegans* animals were cultured on *E. coli* OP50 using standard methods (Sulston and Hodgkin, 1988). The wild-type strain was N2. Mutations: LG X: *hnd-1(q740)*, *med-1(ok804)*. LG I: *unc-120(st364)*. LG II: *tbx-35(tm1789)*, *hlh-1(cc561ts)*. LG III: *unc-119(ed4)*, *med-2(cx9744)*. LG IV: *skn-1(zu67)*. LG V: *ceh-51(tm2123)*. Rearrangement: *nT1 [unc-?(n754) let-?](IV;V)*. Transgenes: *gvIs401 V* [*unc-120*::GFP], *gvIs402 I* [*unc-120*::GFP], *cuIs1 V* [*ceh-22*::GFP], *ccIs7963 V* [*hlh-1*::GFP], *qIs55* [*hnd-1*::GFP], *irIs57 III* [hs-*ceh-51*], *irIs70* [hs-*ceh-51*], *cdIs41 II* [*cup-4*::GFP], *cdIs42 I* [*cup-4*::GFP], *ruIs37 III* [*myo-2*::GFP], *pxIs[pha-4*::GFP*] IV*, *irIs39 III* [*ceh-51*::GFP], *irIs41* [*ceh-51*::GFP], *irIs42 X* [*hs-tbx-35*], *irIs58* [*hs-ceh-51*], *irIs89* [*ceh-51(+)*], *qtIs9* [*nhr-25*::YFP]. We have previously observed a lack of strict additivity and variability in the number of cells expressing tissue-specific reporters (Lin et al., 2009). We attribute this primarily to expression mosaicism between animals and the difficulty of resolving adjacent cells.

### Identification of *ceh-51*

Y80D3A.3 (previously *dlx-1*) was named *ceh-51* in consultation with Thomas Burglin and Jonathan Hodgkin (Karolinska Institute, Stockholm, Sweden and University of Oxford, Oxford, UK). *ceh-51* resides within intron 12 of Y80D3A.2/*emb-4* (WormBase, WS200 release). Four ESTs support a single transcript with one intron for *ceh-51* that does not overlap *emb-4* exonic sequences (Kohara, 2001). As RNAi targeted to introns does not affect mature transcripts (Fire et al., 1998), it is unlikely that RNAi targeted to *ceh-51* would affect transcripts of *emb-4*. Indeed, RNAi of *emb-4* results in embryonic lethality (Katic and Greenwald, 2006), not larval arrest (see text).

### Construction of *ceh-51(tm2123)* strains

We injected a heterozygous *ceh-51(tm2123)* strain (a gift from Shohei Mitani, National Bioresource Project, Japan) with overlapping genomic PCR products spanning the *ceh-51* locus (but lacking any exonic *emb-4* sequences; primer sites are shown in Fig. 2) and an *unc-119*::CFP reporter (pMM809) to produce MS1206, a line that segregated arrested larvae and *unc-119*::CFP(+) viables. We confirmed the correct splicing of *emb-4* in the *tm2123* strain by RT-PCR. After backcrossing, the array was replaced with another carrying *ceh-51(+)*, *unc-119*::mCherry (pMM824) and *myo-2*::mCherry (pCFJ90) for the muscle phenotype synergy experiments. PCR confirmed homozygosity of the *tm2123* deletion in this strain. A

spontaneous integrant of a *ceh-51(+)* array, *irIs89*, showed that 96% (*n*=253) of *ceh-51(tm2123); irIs89* embryos were rescued to full viability. A *tbx-35(tm1789); ceh-51(tm2123)* double mutant strain was made by crossing *tbx-35; Ex[tbx-35(+), unc-119*::YFP*]* males to *ceh-51; Ex[ceh-51(+), unc-119*::CFP*]* hermaphrodites, and identifying YFP/CFP-expressing $F_2$ animals that gave arrested embryos/larvae and in which all viable animals expressed both YFP and CFP. The two arrays in MS1275 were replaced by a single array marked with *unc-119*::mCherry (pMM824) or *sur-5*::dsRed (pAS152).

### Cloning and transgenics

To construct *ceh-51*::GFP (pGB196), a PCR product containing 788 bp upstream of the *ceh-51* start codon and 204 bp of the coding region was cloned into the *Sph*I-*Bam*HI sites in pPD95.67. A smaller reporter, with 187 bp of upstream DNA and 5 bp of coding region, was cloned similarly (pWH270). TBX-35 sites were mutated into restriction sites by PCR in pWH270. A translational fusion was constructed by combining 358 bp of *ceh-51* promoter, a GFP coding region from pPD95.67 and the genomic region of *ceh-51* containing the exons, intron and 3′UTR. A heat-shock *ceh-51* construct was created by cloning the coding region, intron and 468 bp of the 3′UTR into pPD49.78. Further PCR and cloning details are available on request. Transgenics and integrants were made as described (Maduro et al., 2001).

### RNAi experiments

For feeding-induced RNAi, L4 animals were fed for 36 hours on *E. coli* HT115 from the OpenBioSystems RNAi Library or transformed with clones made in pPD129.36. Adults were transferred to fresh plates for egg laying for 4-6 hours at 25°C. Embryos were allowed to develop for 12-24 hours prior to scoring. For dsRNA synthesis, PCR products carrying the T7 RNA polymerase recognition sequence at each end were amplified from N2 DNA, cDNA clones or the Ahringer Lab RNAi Library (Kamath and Ahringer, 2003). dsRNA was synthesized using the Ambion MEGAscript T7 Kit and microinjected into late L4 worms or young adults as described (Ahringer, 2006). Injected animals were allowed to recover for 3-24 hours and transferred to fresh plates for egg laying.

### In situ hybridization

Embryos were stained as described (Coroian et al., 2005). For *pal-1* staining of *med-1,2* and *ceh-51; tbx-35* embryos, a mixture of rescued and non-rescued embryos were stained, and the number of mutants was estimated from the array transmission frequency.

### Phalloidin staining

Embryos or larvae were freeze-cracked on dry ice or frozen in liquid nitrogen, fixed in 4% formaldehyde and stained with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) as described (Shaham, 2006).

### Laser ablation, microscopy and imaging

Laser ablations were performed as described (Lin et al., 2009). Animals were imaged on a Zeiss Axioplan using a Hamamatsu ORCA II digital camera, or on an Olympus BX-61 with a Canon 350D camera. For phalloidin-stained larvae, a Zeiss LSM510 confocal microscope was used (Microscopy and Imaging Core Facility, UC Riverside). Adobe Photoshop 7 and ImageJ v1.37 were used to adjust image brightness and generate overlays.

### Heat-shock experiments

Embryos were heat shocked as a group for 30-45 minutes at 33°C while they were contained within hermaphrodite mothers, representing a developmental time interval of 0-3 hours. After heat shock, hermaphrodites were allowed to lay eggs for 3-4 hours. Embryos were allowed to develop for a further 6-12 hours before scoring. For in situ hybridizations after heat shock, mothers were left overnight at 15°C on plates without food.

### Expression and purification of recombinant TBX-35

A cDNA fragment encoding amino acids 120-325, corresponding to the predicted TBX-35 DNA-binding domain, was cloned into the GST vector pGEX-4T-1 (GE Healthcare) to generate pWH173. This was transformed into *E. coli* Rosetta2 cells (Novagen), grown at 37°C to an OD of 0.3, and protein production was induced with 0.3 mM IPTG overnight at 25°C. Cells were resuspended in BugBuster HT (Novagen) with one tablet of Complete, Mini, EDTA-free Protease Inhibitor (Roche). Glutathione beads, swelled in phosphate-buffered saline (PBS), were added to the lysate for 1 hour. After three washes with PBS, the protein was eluted in 50 mM Tris-HCl (pH 7.5), 5 mM reduced glutathione, 80 mM NaCl, 0.03% Triton X-100, and desalted using a P6 column (BioRad). The protein was stored at –20°C in 50% glycerol with 1 mM DTT and 10 mM Tris (pH 7.5).

### Gel shift and DNase I footprinting

EMSA probes were gel-purified PCR products generated with a $^{32}$P end-labeled primer and an unlabeled primer. The probes contained DNA corresponding to –187 bp to +5 bp relative to the *ceh-51* ATG. Probes carrying mutated sites were amplified from the corresponding GFP reporters. Gel shift and DNase I footprinting were performed as previously described for MED-1 (Broitman-Maduro et al., 2005), except that 10 μM GST and 10, 25 and 50 μM GST::TBX-35(DBD) were used, 6% acrylamide gels were run, and complexes were treated with 0.5 units of DNase I (Epicentre) for 40 seconds prior to organic extraction. For competition arrays, complementary oligonucleotides were annealed at 95°C for 5 minutes, cooled for 15 minutes and added to reactions at a 50-fold excess.

## RESULTS

### Identification of CEH-51, a putative NK-2 class homeodomain transcription factor

Loss of *med-1,2* leads to a highly expressive loss of MS-derived tissues, whereas loss of *tbx-35* has a less expressive MS phenotype, especially at lower temperatures (Broitman-Maduro et al., 2006; Maduro et al., 2001) (this work), suggesting that an additional factor contributes to MS specification downstream of MED-1,2 (Broitman-Maduro et al., 2006). From embryonic transcriptome analyses (Baugh et al., 2005; Baugh et al., 2003), we identified Y80D3A.3 as a candidate early MS lineage gene. Transcripts were reported to accumulate when the MS lineage is undergoing its first divisions, and were reduced in *mex-3(zu155); skn-1(RNAi)* embryos, which do not correctly specify MS. In parallel, we identified Y80D3A.3 in an RNAi screen for enhancement of *hlh-1(cc561ts)* muscle defects (S.K. and P.W.S., unpublished results).

The Y80D3A.3 gene encodes a putative homeodomain transcription factor, CEH-51 (Fig. 2). Of the 89 homeodomain proteins encoded by the *C. elegans* genome (Okkema and Krause, 2005), CEH-51 is most closely related to CEH-7 (Kagoshima et al., 1999), CEH-24 (Harfe and Fire, 1998) and TAB-1 (CEH-29) [L. Carnell and M. Chalfie, unpublished data cited in Syntichaki and Tavernarakis (Syntichaki and Tavernarakis, 2004)], sharing 41-48% identity (57-58% similarity) within the homeodomain (Fig. 2B,C). The CEH-51 homeodomain is most closely related to those of NK-2 subfamily proteins, with which it shares 39-43% identity (59-62% similarity), although CEH-51 lacks the conserved tyrosine at position 54 of the homeodomain (asterisk in Fig. 2C) that is typical of NK-2 proteins (Harvey, 1996). The *C. elegans* pharynx muscle NK-2 factor CEH-22 is more closely related to other NK-2 family members, as it contains the conserved tyrosine and shares 85% identity (90% similarity) with *Drosophila* Vnd/NK-2 across the homeodomain. CEH-51 contains multiple serine residues in its N-terminus (16/50 residues), a feature noted for the N-termini of CEH-24 (Harfe and Fire, 1998) and the endoderm-specifying END-1,3 GATA factors (Maduro et al., 2005b).

### *ceh-51* is expressed in the early MS lineage downstream of TBX-35

We confirmed that *ceh-51* transcripts accumulate in the MS daughters and persist into the MS granddaughters, as observed in 91% (*n*=70) of embryos at the $MS^2$ to $MS^4$ stage (Fig. 3A,B).

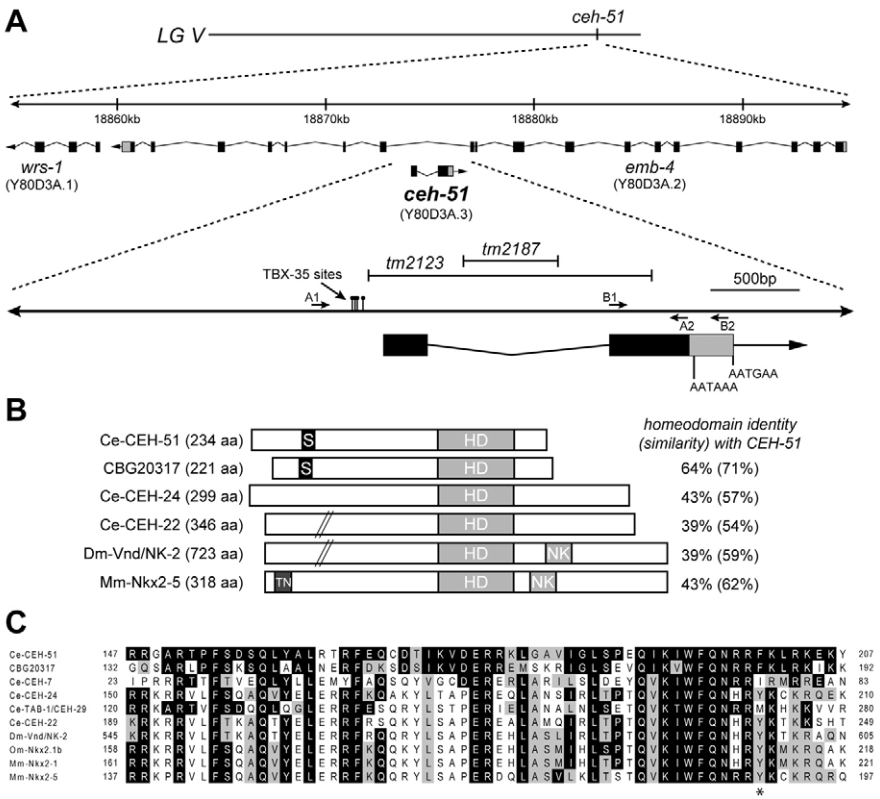



**Fig. 2. Structure of *ceh-51* and its gene product.** (**A**) Location of *ceh-51* and *emb-4* exons on LG V. The locations of the mutant alleles *tm2123* and *tm2187* and the primer pairs A1/A2 and B1/B2 (used to generate overlapping PCR products for rescue of *tm2123*) are shown. A 3′UTR of ~260 bases is predicted by EST yk51g7. Polyadenylation motifs of AATAAA and AATGAA (Hajarnavis et al., 2004) are found 40 bp and 260 bp, respectively, downstream of the stop codon. *tm2123* is a 1610 bp deletion that includes the coding portion of exon 1 and part of exon 2, including the first six amino acids of the predicted homeodomain, and carries an additional 14 bp insertion. The remainder of the *ceh-51* coding region in *tm2123* lacks any in-frame ATG codons, suggesting that *tm2123* is null. *tm2187* is an intronic 540 bp deletion and was not studied. (**B**) Comparison of CEH-51 and other NK-2 proteins. Like all *C. elegans* NK-2 factors, CEH-51 lacks the Tinman (TN) and NK-2-specific (NK) domains that are found in many other NK-2 factors (Harvey, 1996). Regions where at least 7/10 contiguous residues are serine are indicated by S. HD, homeodomain. (**C**) Homeodomain alignments. Identities with *C. elegans* CEH-51 are indicated by black boxes and similarities by gray boxes. A tyrosine residue found in NK-2 family members is indicated with an asterisk (Harvey, 1996). Accession numbers: *C. elegans* (Ce) CEH-51, CAB60440; CEH-7, AAC36745; CEH-24, AAB81844; TAB-1 (CEH-29), AAA98021; CEH-22, NP_001076744; *C. briggsae* CBG20317, CAP37360; *Oncorhynchus mykiss* (Om) Nkx2.1b, BAD93686; *Mus musculus* (Mm) Nkx2.1, NP_033411; Nkx2.5, NP_032726; *Drosophila melanogaster* (Dm) Vnd, P22808.

Similar expression was seen with a *ceh-51*::GFP transcriptional reporter carrying 788 bp of genomic DNA upstream of the predicted ATG (Fig. 3E), a GFP::CEH-51 translational fusion with 358 bp of upstream region (Fig. 3F), and from expression reported by others (Hunt-Newbury et al., 2007; Kohara, 2001; Reece-Hoyes et al., 2007). As anticipated by the mis-specification of MS in *skn-1* and *med-1,2* mutant embryos (Bowerman et al., 1992; Maduro et al., 2001), expression of *ceh-51*::GFP was not observed in these backgrounds (Fig. 3G; data not shown). Conversely, ectopic *ceh-51*::GFP was observed in *mex-1* and *pie-1* RNAi backgrounds (Fig. 3I,J), in which additional MS-like cells are made from the AB and C lineages, respectively (Mello et al., 1992). We have previously found that *tbx-35* is still expressed in MS in a *pop-1(RNAi)* background (Broitman-Maduro et al., 2006), even though in this background MS adopts an E-like fate (Lin et al., 1995). Unexpectedly, most *pop-1(RNAi)* embryos expressed *ceh-51* in both the MS and E lineages (Fig. 3D,H).

The expression pattern of *ceh-51* suggests that it is a direct target of TBX-35. Overexpression of TBX-35 was sufficient to cause ectopic *ceh-51* activation (Fig. 3C), whereas weaker expression still occurred in approximately half of *tbx-35(tm1789)* mutants (Fig. 3K), demonstrating that TBX-35 is sufficient but not necessary for *ceh-51* activation. In a *tbx-35(tm1789); pop-1(RNAi)* background, expression of *ceh-51*::GFP became undetectable (Fig. 3L), suggesting that activation of *ceh-51* in a *tbx-35* mutant background is POP-1-dependent.

To test for direct interaction of TBX-35 with *ceh-51*, we purified recombinant GST::TBX-35 DNA-binding domain (DBD) expressed in *E. coli*, and found that a 187 bp fragment of *ceh-51* could be gel shifted (Fig. 4A, lanes 6-8). We identified four putative TBX-35 binding sites based on similarity to the consensus sequence for the founding T-box factor Brachyury (Kispert and Herrmann, 1993), and confirmed that they were protected in a DNase I footprinting assay (Fig. 4B). These regions define a consensus of RTSKCACCYNNYY (Fig. 4C), which matches 7/8 sites of the Brachyury half-site TCACACCT (matches underlined) (Kispert and Herrmann, 1993). Hence, it is likely that TBX-35 binds DNA as a monomer, similar to mouse Tbx20 and Tbx5 (Ghosh et al., 2001; Macindoe et al., 2009; Stennard et al., 2003). A competitor oligonucleotide containing two of the candidate sites competed the shifts, whereas a competitor with both sites mutated did not (Fig. 4A, lanes 9-11), and all four sites appear to be important for TBX-35

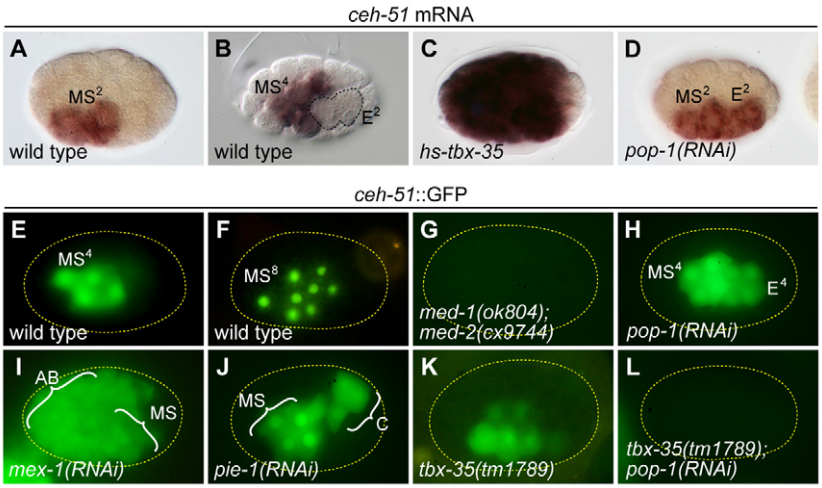



**Fig. 3. Expression of *ceh-51*.** (**A**,**B**) *ceh-51* transcripts occur in (A) the MS daughter cells ($MS^2$) and (B) in the MS granddaughters ($MS^4$), as detected by in situ hybridization. The E daughters are outlined. Ninety-one percent (*n*=101) of embryos at this stage showed expression in $MS^2$ or $MS^4$ (nine embryos did not stain). (**C**) Ectopic expression of *ceh-51* following heat shock of hs-*tbx-35* embryos. (**D**) Eighty-six percent (*n*=44) of *pop-1(RNAi)* embryos showed *ceh-51* mRNA in both the MS and E daughters. Two embryos showed normal expression and four embryos did not stain. (**E**) Embryos transgenic for a *ceh-51*::GFP transcriptional reporter with 788 bp of upstream sequence show expression at $MS^4$ that persists in later MS descendants. (**F**) A translational *ceh-51*::GFP::CEH-51 fusion shows strong nuclear accumulation at $MS^8$. (**G**) *ceh-51*::GFP is undetectable in *med-1(ok804); med-2(cx9744)* embryos (*n*=84). (**H**) Sixty-six percent (*n*=41) of *pop-1(RNAi)* embryos showed *ceh-51*::GFP in both the MS and E lineages (the remainder were similar to wild type). (**I**,**J**) *mex-1(RNAi)* (I) and *pie-1(RNAi)* (J) embryos displayed ectopic *ceh-51*::GFP in AB and C descendants. (**K**) In *tbx-35(tm1789)* embryos, the onset of *ceh-51*::GFP expression was undetectable (52%, *n*=89) or delayed until past the $MS^8$ stage (48%) and at lower levels. The exposure in this image was 10-fold longer than that shown in E. (**L**) *ceh-51*::GFP was not detected in *tbx-35(tm1789); pop-1(RNAi)* embryos (*n*=49).

binding (Fig. 4D). In vivo, a minimal *ceh-51*::GFP reporter carrying the four sites was expressed in the early MS lineage, its expression was abolished in a *tbx-35(tm1789)* background, and mutation of the sites resulted in a loss of expression (Fig. 4E). We conclude that TBX-35 directly activates *ceh-51*.

## Overexpressed CEH-51 is sufficient to promote aspects of MS specification

We next assessed the ability of CEH-51 to specify the development of MS-derived cell types using a heat-shock (hs) *ceh-51* transgene. Ninety-one percent (*n*=245) of heat shocked pregastrulation hs-*ceh-51* embryos underwent arrest, whereas heat shock of wild types resulted in only 22% (*n*=243) embryonic arrest. We examined pharynx muscles with *ceh-22*::GFP (Okkema and Fire, 1994), using a *skn-1(RNAi)* background to eliminate MS-derived tissues and AB-derived pharynx (Bowerman et al., 1992). Among *skn-1(RNAi);* hs-*ceh-51* embryos, we observed only a small number of *ceh-22*::GFP-positive cells following heat shock (Fig. 5F), and were unable to detect significant expression of the pharynx identity gene *pha-4* (Horner et al., 1998) or the pharyngeal myosin gene *myo-2* (Miller et al., 1986) (Fig. 5G; data not shown), suggesting that CEH-51 by itself has, at most, a weak ability to specify pharynx.

Next, we examined production of muscle in a *skn-1(RNAi); pal-1(RNAi)* background, which blocks specification of nearly all body muscles (Hunter and Kenyon, 1996). In such embryos, hs-*ceh-51* was sufficient to promote widespread muscle specification as scored by *unc-120*::GFP (Fukushige et al., 2006) and expression of the body muscle gene *myo-3* (Miller et al., 1986) (Fig. 5H,I). Hence, CEH-51 is sufficient to specify muscle cell fate.

We then examined production of the four embryonically derived coelomocytes, which arise fairly late in the MS lineage (Sulston et al., 1983), using *cup-4*::GFP (Patton et al., 2005). hs-*ceh-51* was sufficient to cause specification of coelomocytes in a *skn-1(RNAi)* background, which by itself eliminates them (Table 1; Fig. 5E,F). We conclude that CEH-51 is sufficient to specify muscle and coelomocyte precursors. No attempt was made to optimize the time interval for CEH-51 responsiveness, although under the same conditions, overexpressed *tbx-35* was able to cause specification of pharynx, muscle and coelomocytes (Broitman-Maduro et al., 2006) (data not shown).

## Loss of *ceh-51* function results in defects in MS-derived tissues

To evaluate the requirement for *ceh-51* in MS specification, we examined *ceh-51(RNAi)* and *ceh-51(tm2123)* animals. Gonadal injection of *ceh-51* dsRNA resulted in 47% (*n*=70) of progeny arresting as uncoordinated L1 larvae, whereas the remainder appeared normal (50%) or arrested as early embryos (3%). The putative null mutant, *tm2123* (Fig. 2A), resulted in a fully penetrant recessive zygotic L1 arrest. This lethality could be rescued by a *ceh-51(+)* transgene (see Materials and methods).

We examined *ceh-51* mutants for pharynx defects. *ceh-51(tm2123)* mutants had a poorly defined metacorpus and an incompletely developed grinder (Fig. 6A,D), and expression of the pharynx muscle reporter *ceh-22*::GFP (Okkema and Fire, 1994) was observed both inside and outside of the pharynx basement membrane, suggesting defective pharynx integrity (Fig. 6B,E). We also observed detachment of the pharynx from the buccal cavity in 64% (*n*=56) of animals. Similar defects were apparent in *ceh-51(RNAi)* arrested larvae (data not shown). *ceh-51(tm2123)* mutants also had defects in the organization of actin filaments as detected by phalloidin staining (Fig. 6C,F). We scored production of all pharynx cells in *ceh-51* mutants using a *pha-4*::GFP reporter (Horner et al., 1998), and found that the number of cells in *ceh-51* mutants

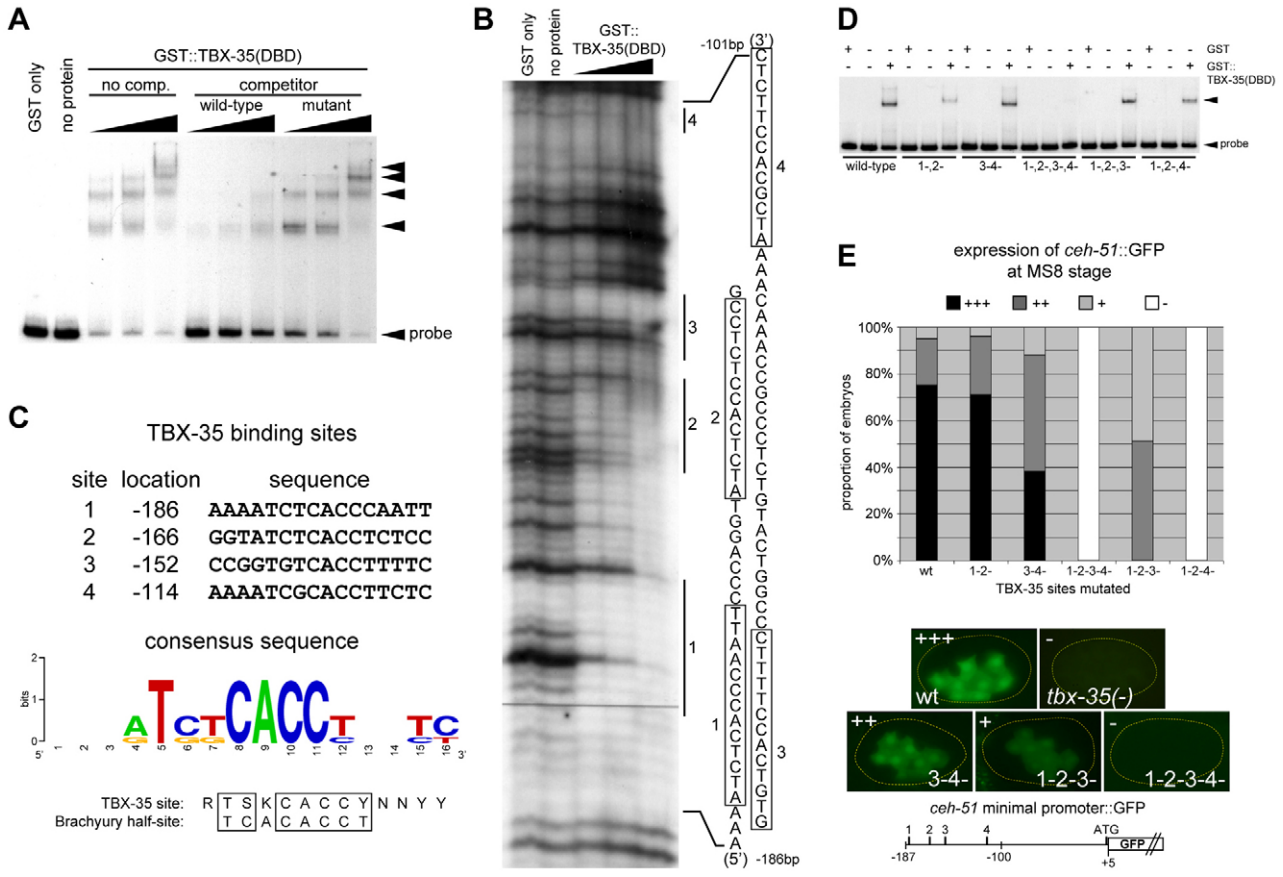



**Fig. 4. TBX-35 binds *ceh-51*.** (**A**) GST::TBX-35(DBD) shifts a *ceh-51* promoter fragment. Competitor oligonucleotides containing two binding sites competed the shift, whereas a mutant competitor did not. (**B**) DNase I footprinting of the *ceh-51* promoter by GST::TBX-35(DBD) at four regions (boxed). The thin horizontal line is the join between two autoradiographs. (**C**) A provisional recognition sequence for TBX-35 (rendered by WebLogo, http://weblogo.berkeley.edu) resembles that of the Brachyury half-site (Kispert and Herrmann, 1993). (**D**) TBX-35-dependent shifting of a 187 bp fragment of the *ceh-51* promoter as demonstrated with a subset of possible binding site mutants. (**E**) Expression of a minimal *tbx-35*-dependent *ceh-51*::GFP reporter in a wild-type background, in *tbx-35(tm1789)*, and in a wild-type background in which the reporter has been mutated at two or more binding sites. At least two transgenic lines and 50 embryos were tested for each promoter.

(47.8±0.9, *n*=17) was similar to that in wild type (50.0±0.9, *n*=21, *P*=0.1). We compared the pharynx defects in *ceh-51* mutants with those of *tbx-35(tm1789)* larvae produced at 15°C. Although such larvae also displayed grinder abnormalities and a defective terminal bulb, they showed a well-defined metacorpus and less disorganized muscle actin, although expression of *ceh-22*::GFP was often mosaic in posterior pharynx muscle cells (Fig. 6G-I). These results suggest that *ceh-51* primarily affects pharynx development and not specification.

Next, we examined the production of body muscles using *unc-120*::GFP (Fukushige et al., 2006). Seventy-five percent (*n*=20) of *ceh-51* embryos at the 1.5-fold stage lacked proper expression in the anterior region of the embryo where MS-derived muscles are normally found (Fig. 7A,D) (Sulston et al., 1983). Mutants frequently displayed additional expression of *unc-120*::GFP displaced slightly to the posterior, suggesting that muscle cells might have migration defects (Fig. 7D). As Caudal/PAL-1 is required for nearly all non-MS body muscles (Hunter and Kenyon, 1996), we scored muscle cells produced in a *pal-1(RNAi)* background in wild type and *ceh-51* mutants using *hlh-1*::GFP (Krause et al., 1994) (Table 1). *ceh-51(tm2123); pal-1(RNAi)* embryos made 19.3±0.5 (*n*=11) *hlh-1*::GFP cells, slightly less than *pal-1(RNAi)* alone (21.6±0.9, *n*=13, *P*=0.02). In the case of *tbx-35(tm1789); pal-1(RNAi)* at 20°C, the number of muscle cells was much lower (5.7±0.5, *n*=40, $P=10^{-4}$).

We looked for further evidence of defects in muscle development by combining *ceh-51(tm2123)* or *ceh-51(RNAi)* with reduction in function of one of three factors, HND-1, HLH-1 and UNC-120, that together define the muscle fate in *C. elegans* (Fukushige et al., 2006). Individual loss of function results in mild impairments in muscle function, but their loss in combination causes a synergistic failure of muscle specification, resulting in a paralyzed, arrested 2-fold (Pat) phenotype (Fukushige et al., 2006; Williams and Waterston, 1994). Loss of *ceh-51* synergistically enhanced the phenotypes of loss of *hlh-1*, *hnd-1* or *unc-120* (Fig. 7G). Individually, RNAi for these factors produced less than 1% Pat embryos, but in a *ceh-51(tm2123)* background, 47% Pat resulted from *hlh-1(RNAi)* and ~10% Pat from *hnd-1(RNAi)* or *unc-120(RNAi)* (e.g. Fig. 7E); a similar result was observed with *ceh-51(RNAi)* (Fig. 7F).

Finally, we observed a decrease in expression of the coelomocyte marker *cup-4*::GFP (Patton et al., 2005), from an average of 3.7±0.2 cells (*n*=105) in wild type to 2.1±0.1 (*n*=53) in *ceh-51(tm2123)* (Table 1), further suggesting that the development of MS

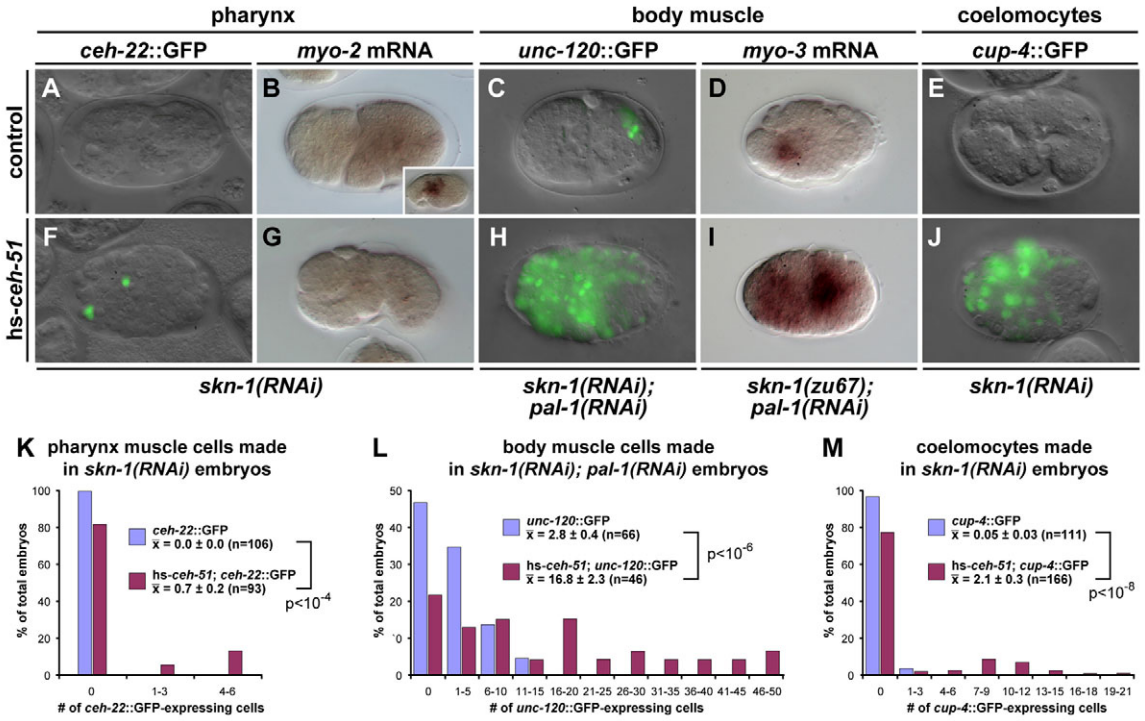



**Fig. 5. Overexpression of CEH-51 promotes specification of MS-derived cell types.** (**A**,**F**) A small number of cells expressing *ceh-22*::GFP are restored to *skn-1(RNAi)* embryos by hs-*ceh-51*. (**B**,**G**) Expression of the pharynx muscle gene *myo-2* is largely absent in both *skn-1(RNAi)* and *skn-1(RNAi)*; hs-*ceh-51* embryos. The inset in B shows wild-type expression of *myo-2*. (**C**,**H**) Many hs-*ceh-51* embryos display *unc-120*::GFP-expressing cells in a *skn-1(RNAi); pal-1(RNAi)* background, which depletes embryos of nearly all body muscles (Hunter and Kenyon, 1996). (**D**,**I**) In a *skn-1(zu67); pal-1(RNAi)* background, heat shock of *ceh-51* causes the generation of many cells expressing the muscle myosin gene *myo-3*. One hundred percent (*n*=79) of heat shocked non-transgenic embryos resembled those shown in D, whereas 53% (*n*=53) of heat shocked transgenics resembled those shown in I. (**E**,**J**) hs-*ceh-51* embryos accumulate ectopic coelomocytes. (**K-M**) Bar charts summarizing the hs-*ceh-51* data.

descendants is compromised in mutants. Taken together, these results show that CEH-51 is required for the normal development of multiple MS tissue types.

## TBX-35 and CEH-51 have overlapping function

Loss of *med-1,2* results in an embryonic lethal phenotype in which arrested embryos elongate to between one and two times the length of the eggshell (Maduro et al., 2007; Maduro et al., 2001). By contrast, *tbx-35* null mutants arrest with varying degrees of elongation, ranging from 1-fold to complete elongation and hatching (Broitman-Maduro et al., 2006). These results suggest that TBX-35 works with another factor. Two further observations support this notion. First, whereas *med-1(ok804); med-2(cx9744)* double mutants and *skn-1(RNAi)* embryos made less than 0.2 coelomocytes per embryo (Table 1; Fig. 8L), *tbx-35(tm1789)* embryos raised at 15°C made as many coelomocytes (3.8±0.2, *n*=28) as wild types (3.7±0.2, *n*=105, *P*>0.9). Second, *tbx-35(tm1789)* embryos achieved further elongation overall when raised at 15°C (Fig. 8P). This increased elongation correlated with an increase in production of MS-derived pharynx cells as scored by *pha-4*::GFP in a *glp-1(RNAi)* background, which eliminates AB-derived pharynx (Priess et al., 1987) (Table 1): *tbx-35; glp-1(RNAi)* embryos at 15°C made 6.6±0.5 pharynx cells (*n*=23), whereas at 23°C only 1.1±0.3 cells were made (*n*=32, $P<10^{-11}$).

We hypothesized that *tbx-35* and *ceh-51* double mutants might show a stronger phenotype than either single mutant, given that *ceh-51* is still activated in *tbx-35(tm1789)* (Fig. 3K). As shown in Fig. 8 and Table 1, *ceh-51(tm2123); tbx-35(tm1789)* double mutants displayed phenotypes that are indistinguishable from *med-1(ok804); med-2(cx9744)* (henceforth abbreviated as *ceh-51; tbx-35* and *med-1,2*). First, *ceh-51; tbx-35* double mutants displayed a strong embryonic arrest that is not temperature sensitive (*P*=0.48 for 15°C versus 20°C) (Fig. 8P) and which is comparable to that of *med-1,2* double mutants at both temperatures (*P*=0.36 and *P*=0.43 for 15°C and 20°C, respectively). Second, development of MS-derived pharynx was eliminated in *ceh-51; tbx-35* (Table 1; Fig. 8E,F), even at 15°C, at which single *ceh-51* and *tbx-35* mutants each displayed a partial grinder (Fig. 6D,G). Using *glp-1(RNAi)* to eliminate AB-derived pharynx, both *med-1,2; glp-1(RNAi)* and *ceh-51; tbx-35; glp-1(RNAi)* embryos made similarly low numbers of pharynx cells (less than two) as scored with *pha-4*::GFP or *ceh-22*::GFP (*P*=0.15 and *P*=0.3) (Table 1). Production of *pal-1*-independent body muscle cells was reduced in *ceh-51; tbx-35; pal-1(RNAi)* embryos to levels comparable to *med-1,2; pal-1(RNAi)* (*P*=0.9) (Table 1; Fig. 8H,I). Lastly, whereas single *ceh-51* and *tbx-35* mutants made reduced numbers of *cup-4*::GFP(+) cells, the double mutants displayed a synergistic reduction similar to that of a *med-1,2* background (*P*=0.04) (Table 1; Fig. 8K,L).

MS adopts a C-like fate in *med-1,2(RNAi)* and *skn-1* mutant embryos (Bowerman et al., 1992; Maduro et al., 2001), but this transformation is weaker in *tbx-35* mutants as zygotic activation of *pal-1* in the MS lineage, a marker of transformation of MS to C (Baugh et al., 2005), was detected in only ~30% of embryos (Broitman-Maduro et al., 2006). We found that 75% (*n*=20) of *med-*

**Table 1. MS-dependent tissues produced in wild-type and mutant embryos**

| Genotype | Pharynx cells† (*pha-4*::GFP) | Pharynx muscles‡ (*ceh-22*::GFP) | Muscle cells (*hlh-1*::GFP) | Coelomocytes (*cup-4*::GFP) |
|---|---|---|---|---|
| Wild type | 50.0±0.9 (21) | 12.8±0.1 (37) | 44.7±1.1 (20) | 3.7±0.2 (105) |
| *skn-1(RNAi)* | 4.8±0.4 (20) | 0.0±0.0 (165) | nd | 0.15±0.04 (124) |
| *pal-1(RNAi)* | 49.5±0.8 (10) | 11.7±0.3 (12) | 21.6±0.9 (13) | 3.7±0.1 (103) |
| *pop-1(RNAi)* | nd | nd | nd | 0.0±0.0 (50) |
| *glp-1(RNAi)* | 23.1±0.6 (15) | 5.7±0.2 (38) | nd | nd |
| *tbx-35(tm1789)* 15°C | 40.6±1.2 (17) | 5.9±0.3 (24) | 37.3±1.6 (10) | 3.8±0.2 (28) |
| *tbx-35(tm1789)* 23°C | 35.7±0.8 (16)** | 5.2±0.2 (46)* | 34.8±2.4 (10) | 3.3±0.4 (20) |
| *tbx-35(tm1789); glp-1(RNAi)* 15°C | 6.6±0.5 (23) | 2.0±0.4 (26) | nd | nd |
| *tbx-35(tm1789); glp-1(RNAi)* 23°C | 1.1±0.3 (32)** | 1.0±0.2 (39)* | nd | nd |
| *tbx-35(tm1789); pal-1(RNAi)* 15°C | 38.8±0.7 (15) | 5.1±0.3 (14) | 8.4±1.0 (17) | 2.2±0.2 (47) |
| *tbx-35(tm1789); pal-1(RNAi)* 23°C | 35.6±1.0 (14)* | 4.7±0.3 (17) | 5.7±0.5 (40)* | 0.6±0.1 (49)** |
| *ceh-51(tm2123)* | 47.8±0.9 (17) | 9.2±0.2 (10) | 42.4±1.4 (10) | 2.1±0.1 (53) |
| *ceh-51(tm2123); pal-1(RNAi)* | nd | nd | 19.3±0.5 (11) | 2.5±0.1 (84) |
| *med-1(ok804); med-2(cx9744)* | 31.3±0.6 (26) | 4.1±0.2 (32) | 31.0±2.7 (10) | 0.07±0.03 (34) |
| *ceh-51(tm2123); tbx-35(tm1789)* | 30.2±0.5 (44) | 4.4±0.2 (18) | 30.1±1.0 (14) | 0.19±0.04 (124)* |
| *med-1(ok804); med-2(cx9744); glp-1(RNAi)* | 1.4±0.4 (14) | 0.3±0.1 (31) | nd | nd |
| *ceh-51(tm2123); tbx-35(tm1789); glp-1(RNAi)* | 1.9±0.5 (26) | 0.5±0.1 (52) | nd | nd |
| *med-1(ok804); med-2(cx9744); pal-1(RNAi)* | nd | nd | 3.8±0.5 (13) | nd |
| *ceh-51(tm2123); tbx-35(tm1789); pal-1(RNAi)* | nd | nd | 3.9±0.4 (15) | nd |

Strains were grown at 20-23°C unless otherwise indicated. Data are shown as the mean ± s.e.m. *0.01<*P*<0.05, **P<0.01, Student's *t*-test, by comparison with the experiment immediately above. nd, not done.
†Only pharynx expression of *pha-4*::GFP, anterior to the gut (when present), was scored.
‡The anatomy of the pharynx was considered in assigning expression to particular muscle cells.

*1,2(–)* and 60% (*n*=35) of *ceh-51; tbx-35* embryos showed ectopic zygotic *pal-1* mRNA in the early MS lineage (*P*>0.3) (Fig. 8N,O). We examined the fate of MS descendants in *tbx-35; ceh-51* double mutants carrying a reporter fusion for *nhr-25*, a C-lineage gene that is expressed in hypodermal precursors and their descendants (Baugh et al., 2005), using a laser to ablate all other cells. Partial embryos resulting from isolated wild-type MS blastomeres failed to show significant *nhr-25*::YFP (*n*=3), whereas 9/9 MS blastomeres from *tbx-35; ceh-51* double mutants, and 5/5 isolated C blastomeres from wild types, generated *nhr-25*::YFP descendants. Hence, *ceh-51; tbx-35* embryos show a strong transformation of MS to C, suggesting that CEH-51 and TBX-35 together account for the majority of normal MS lineage development downstream of MED-1,2.

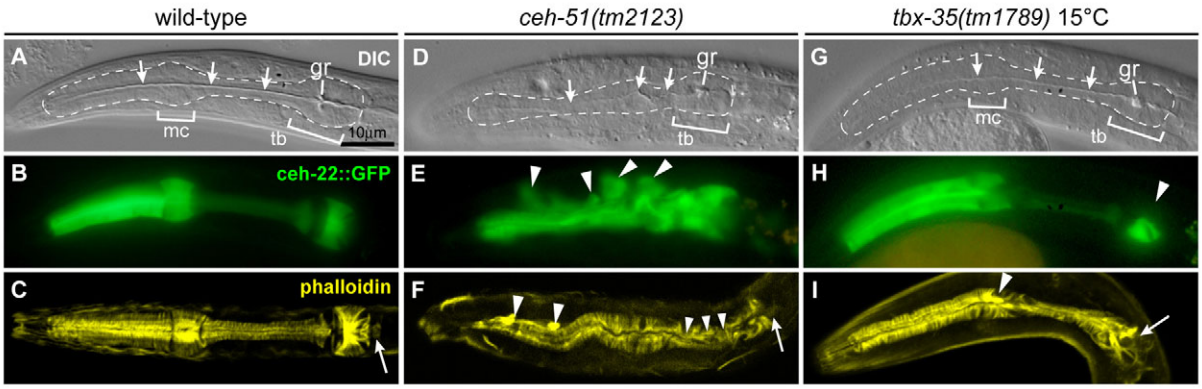
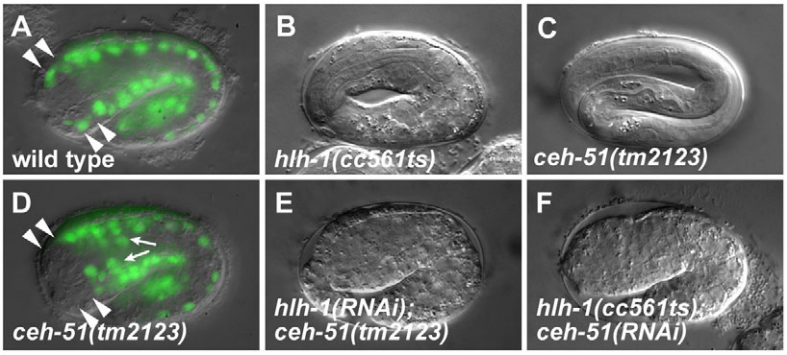



**Fig. 6. *ceh-51* mutants and *tbx-35* mutants raised at 15°C arrest as larvae with pharynx structural defects.** Pharynxes were visualized by DIC optics (A,D,G), *ceh-22*::GFP expression (B,E,H) (Okkema and Fire, 1994) or phalloidin staining (C,F,I) (Franks et al., 2006). In the DIC panels, the lumen (arrows), grinder (gr), metacorpus (mc) and terminal bulb (tb) are indicated and the pharynx is outlined (dashed line). (**A-C**) Wild-type pharynx. (**D-F**) *ceh-51(tm2123)* pharynxes show lumen irregularities and an indistinct metacorpus (D). Protrusions accumulate GFP outside the pharynx, suggesting a defect in pharynx integrity (E). In F, phalloidin staining shows actin filament accumulations (large arrowheads), lumen abnormalities (small arrowheads) and an abnormal terminal bulb (arrow). (**G-I**) *tbx-35(tm1789)* raised at 15°C has a normal lumen but abnormal grinder (G). *ceh-22*::GFP expression (H) shows absence of expression of *ceh-22*::GFP in part of the posterior pharynx (arrowhead); contralateral expression in this region is likely to be in an MS-derived m7 muscle (Okkema and Fire, 1994; Sulston et al., 1983). In I, phalloidin staining shows some actin accumulations (arrowhead) and an abnormal terminal bulb (arrow).




**G**

| genotype | RNA interference: none | *hlh-1* | *hnd-1* | *unc-120* | *ceh-51* |
|---|---|---|---|---|---|
| wild type | 0.5/0.0 n=411 | 5.6/0.0 n=107 | 4.1/0.9 n=222 | 6.6/0.5 n=198 | 8.8/0.6 n=160 |
| *hlh-1(cc561ts)* | 11.7/1.4 n=634 | 25.0/12.5 n=16 | 72.3/49.2 n=65 | 67.3/30.8 n=52 | 98.6/48.6 n=70 |
| *hnd-1(q740)* | 2.0/1.0 n=203 | 86.8/47.4 n=38 | 0.0/0.0 n=121 | 27.5/4.3 n=69 | 47.7/16.2 n=130 |
| *unc-120(st364ts)* | 7.0/0.0 n=86 | 5.9/2.0 n=51 | 83.3/44.4 n=18 | 45.5/4.5 n=22 | 60.0/30.0 n=30 |
| *ceh-51(tm2123)* | 10.8/1.0 n=194 | 73.6/47.2 n=125 | 24.9/9.9 n=213 | 36.2/10.4 n=163 | 32.5/6.6 n=166 |

%Emb / %Pat

**Fig. 7. Muscle defects in *ceh-51(tm2123)*.** (**A**,**D**) Loss of MS-derived *unc-120*::GFP expression (arrowheads) in *ceh-51* (D) as compared with wild type (A). Additional expression is indicated by small arrows. (**B**,**C**,**E**,**F**) Loss of *ceh-51* synergizes with partial muscle specification mutants to produce paralyzed, arrested 2-fold (Pat) embryos. Whereas more than 95% of *hlh-1(cc561ts)* mutants grown at 15°C (B), and more than 99% of *ceh-51(tm2123)* embryos (C), elongated to greater than 3-fold, between 47 and 49% of embryos produced by a combination of mutation of *ceh-51* with RNAi of *hlh-1* (E), or vice versa (F), produced a synthetic Pat phenotype. (**G**) Summary of synthetic Pat phenotypes. Data are shown as the percentage of progeny arresting as embryos (%Emb)/percentage of progeny arresting as paralyzed, 2-fold (Pat) embryos (%Pat) (included in the Emb totals). Backgrounds have been shaded to indicate higher %Pat.

## DISCUSSION

### New regulatory interactions in the MS gene network

We have identified a new regulator, CEH-51, in MS specification. Our results suggest that TBX-35 and CEH-51 could participate in a 'feed-forward' regulatory cascade (Lee et al., 2002), in which TBX-35 activates *ceh-51*, and both TBX-35 and CEH-51 activate common target genes in MS development. There is likely to be at least one other MS lineage activator of *ceh-51* because a *ceh-51*::GFP reporter was still weakly expressed in a *tbx-35* null background (Fig. 3K). Whereas *pal-1(RNAi)* reduced coelomocyte production in *tbx-35(tm1789)* mutants (Table 1), there was no effect on *ceh-51*::GFP expression (data not shown). Instead, this activator appears to be downstream of POP-1 because simultaneous loss of *pop-1* and *tbx-35* resulted in loss of *ceh-51*::GFP expression (Fig. 3L). We also observed ectopic expression of *ceh-51* in the early E lineage in *pop-1(RNAi)* embryos (Fig. 3D,H), suggesting that POP-1 might contribute to repression of *ceh-51* in the E lineage. The observation that a *tbx-35; pop-1* background abolishes all *ceh-51*::GFP expression suggests that ectopic TBX-35 is responsible for E lineage expression of *ceh-51* in *pop-1(RNAi)*. Although we failed to detect activation of *tbx-35* in E in *pop-1(–)* embryos (Broitman-Maduro et al., 2006), such ectopic expression of *tbx-35*::GFP has been observed by others (P. Shetty and R. Lin, personal communication). We have recently shown that in the related nematode *C. briggsae*, POP-1 contributes positively to MS specification in parallel with SKN-1, and there is an apparent function for POP-1 in repression of the MS fate in E (Lin et al., 2009). Hence, these additional roles for POP-1 might be evolutionarily conserved.

### Shared and distinct functions for CEH-51 and TBX-35

Although *ceh-51(tm2123); tbx-35(tm1789)* embryos have a synergistic phenotype compared with the single mutants, each gene has unique essential functions, as evidenced by their distinct phenotypes (Figs 6 and 7). Overexpressed CEH-51 was sufficient to promote specification of muscle and coelomocytes, but was apparently not as effective at promoting pharynx development (Fig. 5), whereas overexpressed TBX-35 could specify all three tissues efficiently (Broitman-Maduro et al., 2006) (data not shown). Conversely, *ceh-51(tm2123)* mutants had only mild defects in pharynx, muscle and coelomocytes, whereas *tbx-35(tm1789)* mutants had strong defects in pharynx and muscle at 20°C (Broitman-Maduro et al., 2006) (Table 1). At 15°C, *ceh-51* is able to partially rescue these defects, resulting in a higher proportion of elongated animals and more normal specification of MS-derived tissues (Table 1). Hence, CEH-51 adds robustness to MS specification primarily at lower temperatures. In the future, identification of TBX-35 and CEH-51 target genes might explain the basis for their different activities, perhaps accounting for why CEH-51 does not rescue aspects of MS specification in *tbx-35* mutants at higher temperatures. We have identified putative TBX-35 binding sites in the promoters of *hlh-1* and *pha-4* (W.W.K.H. and M.F.M., unpublished), although we have not yet identified common targets for both TBX-35 and CEH-51.

### Collaboration of T-box and NK-2 factors in mesoderm development

The apparent collaboration of TBX-35 and CEH-51 in *C. elegans* mesoderm development, downstream of MED-1,2, is highly reminiscent of the roles of related factors involved in cardiac development in other systems. In *C. elegans*, the pharynx is the structure that most closely resembles the heart, as it is a contractile pumping organ that expresses unique sets of myosins (Mango, 2007; Okkema et al., 1993). Expression of vertebrate Nkx2.5 is able to compensate for loss of *ceh-22* in the *C. elegans* pharynx, suggesting a common evolutionary origin of heart and pharynx (Haun et al., 1998). Here, we have shown that TBX-35 and CEH-51 have both distinct and shared roles in pharynx progenitor specification and development. The *Drosophila Nkx2.5* ortholog *tinman* is important for defining early domains that are restricted

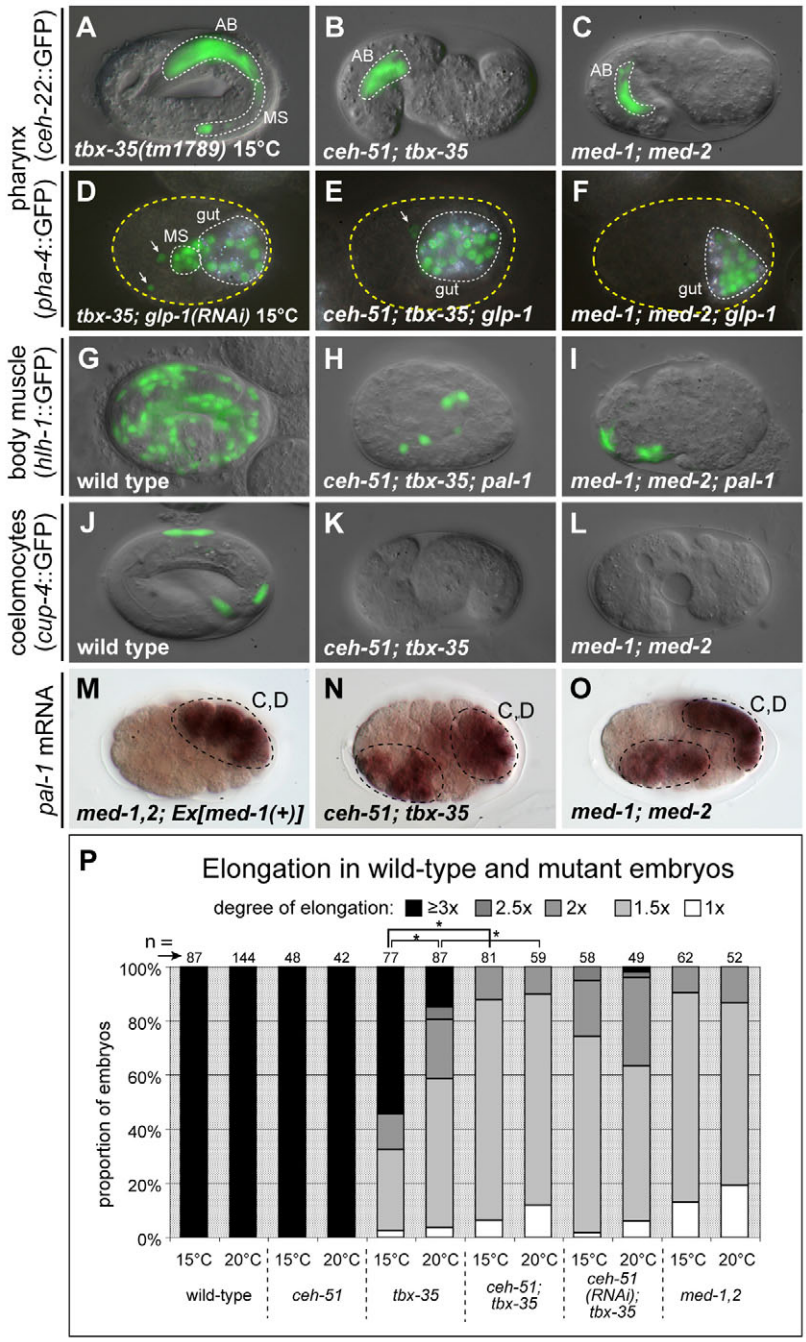



**Fig. 8. Mutation of *ceh-51* and *tbx-35* together synergizes to a *med-1,2(–)* arrest phenotype.** (**A-C**) Pharynx muscles marked by *ceh-22*::GFP (Okkema and Fire, 1994) overlaid on DIC images. (A) Arrested 1.5-fold *tbx-35(tm1789)* embryo raised at 20°C showing AB-derived and MS-derived pharynx muscles. (B) *ceh-51(tm2123); tbx-35(tm1789)* double mutant arrested at ~1.5-fold elongation. (C) *med-1(ok804); med-2(cx9744)* double mutant. (**D-F**) Polarized light images to show gut granules overlaid with *pha-4*::GFP (Horner et al., 1998). (D) At 15°C, *tbx-35(tm1789); glp-1(RNAi)* embryos display 6.6±0.5 (*n*=23) pharynx cells. Some additional GFP-positive cells are seen (arrows); similar 'stray' GFP expression is also seen in a *skn-1(RNAi)* background (see Table 1). Gut/rectum expression of *pha-4*::GFP coincides with birefringence of gut granules, which mark the intestine. (E) *ceh-51(tm2123); tbx-35(tm1789); glp-1(RNAi)* embryo showing a small number of pharynx cells (arrow). (F) *med-1(ok804); med-2(cx9744); glp-1(RNAi)* embryo. (**G-I**) Body muscle cells marked by *hlh-1*::GFP (Krause et al., 1990). (G) Wild-type embryo just before hatching. (H) *ceh-51(tm2123); pal-1(RNAi)* embryo. (I) *ceh-51(tm2123); tbx-35(tm1789); pal-1(RNAi)* embryo. (**J-L**) Coelomocytes marked by *cup-4*::GFP (Patton et al., 2005). (J) Wild-type embryo with four coelomocytes. (K,L) Double *ceh-51(tm2123); tbx-35(tm1789)* or *med-1(ok804); med-2(cx9744)* mutants produce little or no coelomocytes. (**M**) In situ hybridization showing expression of *pal-1* in the early C and D lineages (Baugh et al., 2005). (**N**) Ectopic expression of *pal-1* in *ceh-51(tm2123); tbx-35(tm1789)* double mutant. (**O**) Ectopic *pal-1* in a *med-1(ok804); med-2(cx9744)* embryo. (**P**) Histogram summarizing elongation of wild-type and mutant embryos. *, $P$=0.05 ($\chi^2$ test), for some dataset pairs (comparisons among other pairs are not shown). The total number (*n*) of embryos scored per experiment is shown above each bar.

to forming heart, visceral muscle and some body muscles, as mutants have impairments in the development of these tissues (Azpiazu and Frasch, 1993; Bodmer, 1993). Activation of *tinman* in cardioblasts requires the T-box genes *midline* and *H15* (Reim et al., 2005). In *Xenopus*, the T-box factor Tbx5 is expressed in heart precursors and is known to be essential for heart development (Horb and Thomsen, 1999). Similarly, Nkx2.5 is expressed in early cardioblasts (Lints et al., 1993) and plays an important role in heart patterning, as *Nkx2.5* knockout mice show heart defects (Lyons et al., 1995). Finally, mouse Tbx5 and Nkx2.5 physically interact and collaborate with Gata4/5 in synergistic activation of cardiac genes (Bruneau et al., 2001; Hiroi et al., 2001; Stennard et al., 2003). Hence, the collaboration between TBX-35 and CEH-51 in *C. elegans* might be evolutionarily conserved. Future work aimed at elucidating the gene network downstream of TBX-35 and CEH-51 might uncover further conserved aspects of cardiac and mesoderm development.

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