# Specification of the *C. elegans* MS blastomere by the T-box factor TBX-35

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In *C. elegans*, many mesodermal cell types are made by descendants of the progenitor MS, born at the seven-cell stage of embryonic development. Descendants of MS contribute to body wall muscle and to the posterior half of the pharynx. We have previously shown that MS is specified by the activity of the divergent MED-1,2 GATA factors. We report that the MED-1,2 target gene *tbx-35*, which encodes a T-box transcription factor, specifies the MS fate. Embryos homozygous for a putative *tbx-35*-null mutation fail to generate MS-derived pharynx and body muscle, and instead generate ectopic PAL-1-dependent muscle and hypodermis, tissues normally made by the C blastomere. Conversely, overexpression of *tbx-35* results in the generation of ectopic pharynx and muscle tissue. The MS and E sister cells are made different by transduction of a Wnt/MAPK/Src pathway signal through the nuclear effector TCF/POP-1. We show that in E, *tbx-35* is repressed in a Wnt-dependent manner that does not require activity of TCF/POP-1, suggesting that an additional nuclear Wnt effector functions in E to repress MS development. Genes of the T-box family are known to function in protostomes and deuterostomes in the specification of mesodermal fates. Our results show that this role has been evolutionarily conserved in the early *C. elegans* embryo, and that a progenitor of multiple tissue types can be specified by a surprisingly simple gene cascade.

KEY WORDS: Mesoderm, *C. elegans*, *tbx-35*, Cell fate specification

## INTRODUCTION

The invention of mesoderm marked the defining event in the emergence of triploblastic bilaterally symmetrical animals ~600 million years ago (Chen et al., 2004). Mesoderm in vertebrates includes such tissues as heart, blood, muscles and bone. In the nematode *C. elegans*, the mesodermal cell MS is one of six founder cells that generate the major tissues of the embryo (Fig. 1). MS gives rise to many mesodermal cell types, including cells of the posterior half of the pharynx, one-third of the body muscle cells and four embryonically derived coelomocytes (primitive blood-like cells) (Sulston et al., 1983). MS also signals descendants of the AB lineage, enabling them to make the remaining, largely anterior, half of the pharynx (Priess et al., 1987). Early events that specify MS are tied to the specification of its sister E through a common gene regulatory network that intersects with Wnt/MAPK signaling (Maduro and Rothman, 2002).

The bZIP/homeodomain protein SKN-1, at the top of this pathway, is crucial to the specification of MS and E (Bowerman et al., 1992). *skn-1(–)* embryos fail to specify MS all the time, and E most of the time, and the mis-specified cells adopt the fate of their lineal cousin C, which makes body muscle and hypodermal cells. Ectopic accumulation of SKN-1 results in production of supernumerary MS and E cell types at the expense of others (Bowerman et al., 1993; Bowerman et al., 1992; Mello et al., 1992). At least two activities restrict SKN-1 activity to EMS. In four-cell embryos, SKN-1 protein is detected at high levels in EMS and in its sister cell $P_2$, but at low levels in the two AB daughters ABa and ABp, owing to activity of the CCCH zinc-finger protein MEX-1 (Bowerman et al., 1993). In

$P_2$, PIE-1, also a CCCH zinc finger protein, blocks transcription and, hence, activation of SKN-1 target genes (Batchelder et al., 1999). As shown in Fig. 1, *mex-1(–)* embryos show a transformation of the $AB^4$ cells into MS-like precursors, while in *pie-1(–)* embryos, $P_2$ adopts an EMS-like fate, resulting in a transformation of C to MS and $P_3$ to E (Mello et al., 1992).

To promote specification of MS, SKN-1 activates transcription of the divergent GATA factors *med-1,2* in EMS (Coroian et al., 2005; Maduro et al., 2001). With respect to MS fate, loss of *med-1,2* leads to a similar phenotype as loss of *skn-1* (transformation of MS to C), but although *skn-1* mutant embryos lack pharynx entirely, *med-1,2(–)* embryos still make AB-derived anterior pharynx (Coroian et al., 2005; Maduro et al., 2001). The targets of MED-1,2 in MS that specify a mesoderm fate are not known, although several candidate genes have been identified by bioinformatics and transcriptome analysis (Broitman-Maduro et al., 2005; Robertson et al., 2004).

In the E cell, the MEDs contribute to the activation of the E-specifying genes *end-1,3*, but are dispensable for E specification much of the time (Broitman-Maduro et al., 2005; Goszczynski and McGhee, 2005; Maduro et al., 2001). We have previously reported that 50% of *med-1,2(RNAi)* embryos lack endoderm (Maduro et al., 2001). However, a recent study has shown that zygotic loss of *med-1* and *med-2* results in a weak or undetectable endoderm defect (Goszczynski and McGhee, 2005). We have since found that a significant maternal contribution of the MED genes exists, explaining this discrepancy (M.F.M., G.B.-M., I. Mengarelli and J. Rothman, unpublished). In addition to MED-1,2, we and others have further shown that Caudal/PAL-1 and the Wnt effector TCF/POP-1 also contribute to E specification (Maduro et al., 2005b; Shetty et al., 2005).

The MS and E cells are made different from each other by a molecular switching system that functions in the MS/E decision as well as many other asymmetric cell divisions in *C. elegans* development (Kaletta et al., 1997; Lin et al., 1998). The posterior daughter of EMS is specified to become the endodermal precursor E as a result of a cell-cell interaction between EMS and its sister $P_2$

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(Goldstein, 1992). An 'MS' fate is the default state for an EMS daughter cell, as an isolated EMS cell will divide symmetrically to produce two MS-like cells. Depletion of the components of this signal, an overlapping Wnt/MAPK/Src pathway, results in a similar phenotype in which E adopts an MS-like fate (Bei et al., 2002; Rocheleau et al., 1997; Thorpe et al., 1997). In the MS cell, POP-1 is essential to repress *end-1,3*, as evidenced by the MS to E transformation displayed by *pop-1(–)* embryos (Lin et al., 1995). In the E cell, modification of POP-1 by the WRM-1/LIT-1 kinase blocks the repressive activity of POP-1, allowing POP-1 to become an activator of endoderm (Lo et al., 2004; Rocheleau et al., 1999; Shetty et al., 2005).

We report here that the MED-1,2 target gene *tbx-35*, which encodes a member of the T-box class of transcriptional regulators, specifies the MS fate. Restriction of MED-dependent activation of *tbx-35* to MS results from Wnt-dependent POP-1-independent repression in E, implicating an unknown mechanism that blocks MS fate in the E cell. Our results identify an important regulator in the suite of blastomere-identity genes in the *C. elegans* embryo, and link the mesoderm-specifying activity of *med-1,2* with MS-specific activation of *tbx-35*.

## MATERIALS AND METHODS

### *C. elegans* strains and genetics

Recombinant strains were made by standard crosses. The wild-type strain was N2. The following mutations were used: LG I, *pop-1(zu189)*; LG II, *tbx-35(tm618)*, *tbx-35(tm1789)*; LG III, *glp-1(or178)*, *unc-119(ed4)*, *lit-1(t1512)*. Chromosomally integrated transgenes were: LG V, *cuIs2* [*ceh-22*::GFP], *ccIs7963* [*hlh-1*::GFP], *irIs21* [*elt-2*::YFP]; LG X, *irIs42* [hs-*tbx-35*]. Unmapped integrants were *qtIs12* [*pal-1*::YFP], *irIs3* [*tbx-35*::GFP], *irIs31* [*tbx-35*::GFP] and *mcIs17* [*lin-26*::GFP]. All strains were grown at 20°C with the exception of those containing *glp-1(or178)* or *lit-1(t1512)*, which were propagated at 15°C and tested at 25°C.

The balanced *tbx-35(tm1789)* strain MS516 was made by injecting heterozygotes with a mixture of cosmid ZK177, an *unc-119*::CFP reporter (pMM809) and the *rol-6D* plasmid pRF4 (Mello et al., 1991). The phenotype of *tbx-35(tm1789)* embryos lacking the array persisted after three backcrosses of MS516 to N2. Rescue with a fragment containing only *tbx-35* was obtained by injecting MS516 with a 2.4 kb PCR product containing *tbx-35(+)* and the *unc-119*::YFP::lacZ reporter pMM531, and obtaining lines that had lost the *unc-119*::CFP/Rol array in favor of the *unc-119*::YFP array. PCR was used to validate the genotypes of *tm1789*-derived strains and to confirm the absence of a wild-type copy of *tbx-35* in the *tm618* and *tm1789* strains.

### Microscopy and imaging

All micrographs were obtained on an Olympus BX-71 microscope equipped with a Canon Digital Rebel 350D and LMscope adapter (Micro Tech Lab, Austria). For fluorescence micrographs, images acquired from different focal planes were stacked with Adobe Photoshop 7.

### Plasmids and cloning

A *tbx-35*::GFP reporter was constructed by cloning a 650 bp PCR product, containing 605 bp upstream of the *tbx-35* start codon, into plasmid pPD95.67. A hs-*tbx-35* construct (pGB223) was built by cloning a PCR-amplified genomic fragment containing the coding region and introns into plasmid pPD49.83. A cDNA-derived heat shock construct (pWH95) contained a transversion resulting in an E42V substitution. Expression from this construct was confirmed by in situ hybridization. Oligonucleotide sequences and cloning details are available on request.

### RNA interference (RNAi)

RNAi was performed at 20°C by either direct injection of dsRNA into both gonad arms as reported (Maduro et al., 2001) or by growth on *E. coli* HT115 bacteria engineered to accumulate dsRNA (Timmons and Fire, 1998). For RNAi by injection, embryos were collected starting at 24 hours after parental injection. For RNAi by ingestion, synchronized L2 animals were fed transgenic HT115 for a minimum of 2 days. In our hands, these treatments
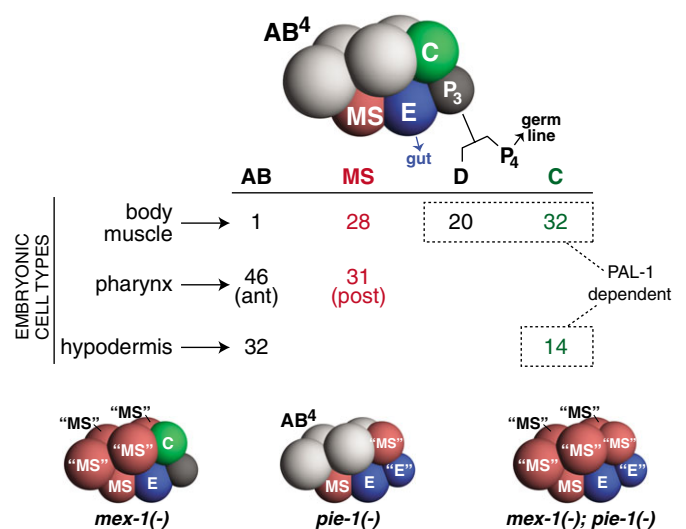


**Fig. 1. Positions and fates of eight-cell stage *C. elegans* embryonic blastomeres in wild-type and mutant backgrounds.** A diagram showing the relative positions of the wild-type AB granddaughters (AB⁴) and the P₁ granddaughters MS, E, C and P₃ is given at the top. Depletion of *mex-1* and *pie-1* individually and together results in the fate transformations shown at the bottom of the figure (Mello et al., 1992). The table summarizing the major embryonic cell types made by descendants of AB, MS, D and C was adapted from lineage data (Sulston et al., 1983). In embryo diagrams, anterior is towards the left, and dorsal is upwards.

produce >99% phenocopy embryos, consistent with published phenotypes. RNAi targeted to multiple genes was performed by injection, controlling for depletion of each targeted gene by parallel single RNAi experiments (Maduro et al., 2001). Control GFP dsRNA injections produced 4% arrested embryos (*n*=591) that did not resemble *tbx-35(tm1789)*. The additional early-arrest embryos seen with *tbx-35(RNAi)* appear to be due to an off-target effect, rather than a maternal contribution: a smaller *tbx-35* dsRNA produced no lethality, no germline transcripts were observed by in situ hybridization and two germline mosaic MS516 mothers produced 100% arrested embryos with the same *tbx-35(–)* phenotypes described (data not shown).

### In situ hybridization

Detection of endogenous mRNAs was performed as described (Coroian et al., 2005).

## RESULTS

### *tbx-35* encodes a divergent T-box gene

We sequenced multiple partial and complete open reading frame RT-PCR products to confirm the ZK177.10/*tbx-35* gene model described in WormBase (release WS156). *tbx-35* encodes a 325 amino acid protein with a Tbx domain near its N-terminal end, and is one of 21 Tbx genes in *C. elegans* (Reece-Hoyes et al., 2005; Wilson and Conlon, 2002). TBX-35 shares 25-28% identity with other metazoan Tbx proteins within the DNA-binding domain (Fig. 2B). The Tbx domain of its closest *C. elegans* homolog, TBX-37, shows more relatedness with other Tbx proteins (32-37% identity), while the more canonical *C. elegans* Tbx protein MAB-9 shares 52% identity across the same region (Woollard and Hodgkin, 2000). Hence, TBX-35 is a fairly divergent Tbx factor.

### *tbx-35* is a direct target of MED-1

Our biochemical analysis of the MED-1 binding sites in *end-1* and *end-3* identified the consensus site RAGTATAC, four of which occur in the *tbx-35* promoter (Fig. 2A) (Broitman-Maduro et al., 2005).

Three additional closely-related RGGTATAC sites, which can bind MED-1 at a lower affinity (our unpublished observations) are also found in close proximity. We and others have previously reported the early MS lineage expression of a *tbx-35*::GFP reporter, as shown in Fig. 3A,B (Broitman-Maduro et al., 2005; Robertson et al., 2004). Using whole-mount in situ hybridization, we confirmed activation

of endogenous *tbx-35* in the MS cell (Fig. 3G). Consistent with positive regulation of *tbx-35*, overexpression of MED-1 is sufficient to promote widespread activation of a *tbx-35* reporter (Broitman-Maduro et al., 2005). SKN-1 protein is also present in MS, but only a single site matching the SKN-1 RTCAT consensus is present in the *tbx-35* promoter, 31 bp upstream of the start codon (Blackwell et al.,
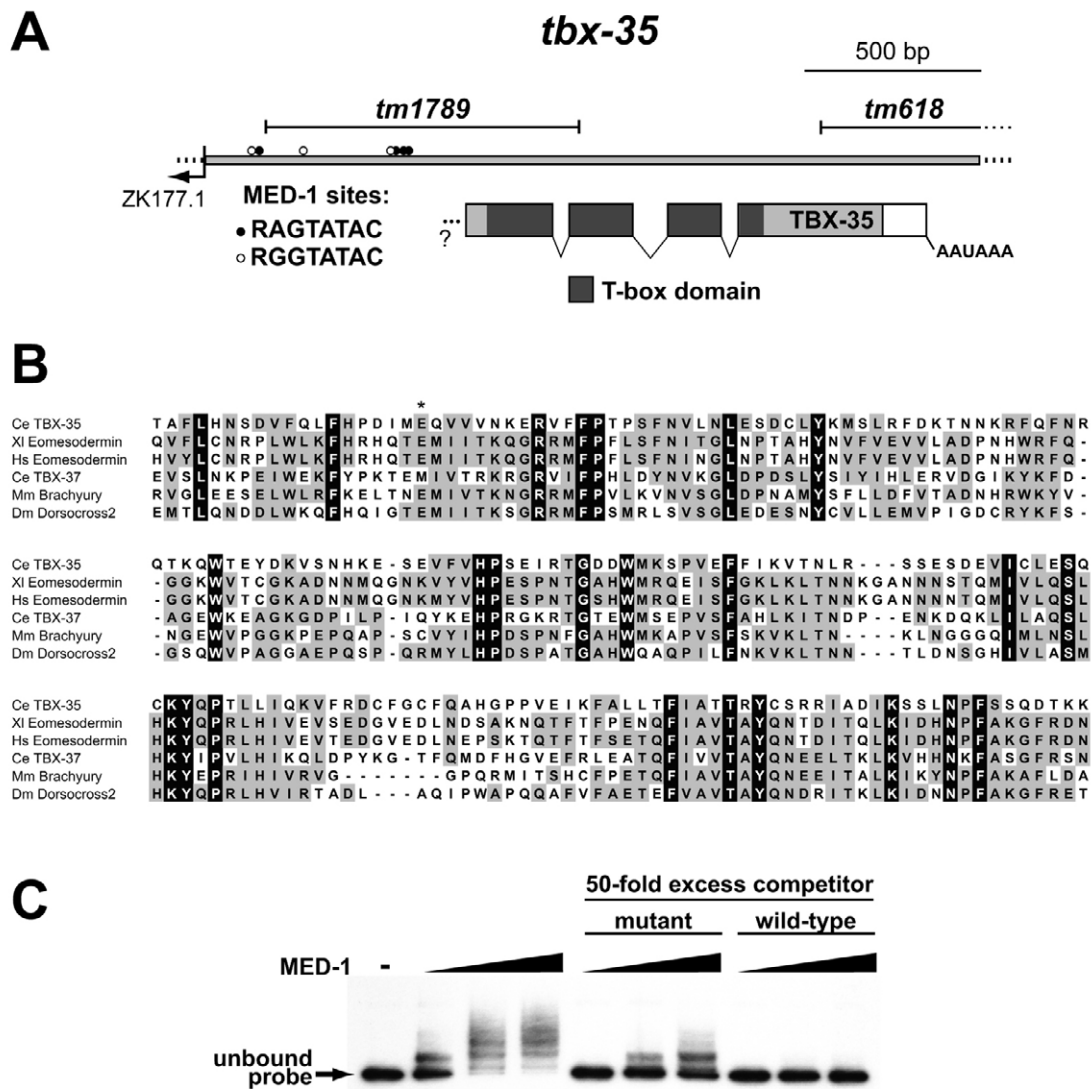


**Fig. 2. The *tbx-35* gene encodes a T-box factor.** (**A**) Gene structure of *tbx-35* on LGII. The left end of the gene is preceded by the start codon of ZK177.1. The deleted regions in *tm1789* and *tm618* are shown above the gene. The predicted mRNA is shown below. The coding region is shaded, with the T-box emphasized. The locations of MED-1 binding sites are shown as grey circles for the RAGTATAC site defined by MED-1 footprinting of the *end-1* and *end-3* promoters (Broitman-Maduro et al., 2005), and white circles for three lower-affinity RGGTATAC sites based on in vitro competition assays (G.B.-M., K.L., W.H. and M.M., unpublished). No additional MED sites are found elsewhere in the gene. We were unable to amplify the 5′ end of the transcript using primers to detect SL1 or SL2, suggesting that the *tbx-35* mRNA is not *trans*-spliced (Conrad et al., 1993); hence, the bona fide 5′ end of the transcript is not known. A polyadenylation sequence (AATAAA) is found 115 bp downstream of the predicted stop codon, but we have not confirmed the precise 3′ end of the transcript. (**B**) Alignment of the conserved T-box region of TBX-35 with those of other Tbx genes. TBX-35 is 25-28% identical (35-39% similar) to vertebrate eomesodermin, mouse brachyury, *Drosophila* Dorsocross2 and its closest homolog in *C. elegans*, TBX-37. The TBX-37 T-box is more closely related to the other T-box regions shown (e.g. 37% identical and 50% similar to brachyury). This alignment was generated with AlignX (within Vector NTI 6) using the default settings. An asterisk indicates a conserved glutamic acid residue that was mutated in a heat shock fusion construct (see text). Xl, *Xenopus laevis*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*. Accession numbers: eomesodermin, P79944 (*Xenopus*) and NP005433 (human); brachyury, CAA35985; Dorsocross2, AAM11545. (**C**) MED-1 binds the *tbx-35* promoter. A 190 bp fragment of the *tbx-35* promoter containing the proximal MED site cluster was incubated with increasing concentrations of recombinant DNA-binding domain of MED-1 as described (Broitman-Maduro et al., 2005). Double-stranded competitor oligonucleotides: wild type, 5′-TCATTTGTATACTTTATCTACAATAT; mutant, 5′-TCATTTGTTATCATTATCTACAATAT-3′. Underlined nucleotides represent the wild-type and mutated core MED-1 binding sites, respectively.

1994; Bowerman et al., 1993). As bona fide SKN-1 targets contain clusters of several overlapping sites in the 5′ flanking region (An and Blackwell, 2003; Coroian et al., 2005; Maduro et al., 2001), it is unlikely that SKN-1 directly regulates *tbx-35*.

Transgenic GFP::MED-1 localizes to extrachromosomal arrays containing a *tbx-35*::lacZ reporter plasmid in vivo, suggesting that MED-1 directly activates *tbx-35* (Broitman-Maduro et al., 2005). We confirmed a direct interaction of *tbx-35* with MED-1 in vitro by gel shift assay using the MED-1 DNA-binding domain (DBD) and



**Fig. 3. Expression of *tbx-35* is specific for the early MS lineage.** (**A,B**) A reporter *tbx-35*::GFP transcriptional fusion shows GFP fluorescence in the two daughters of MS (MSa and MSp) and their descendants for several divisions. (**C**) Depletion of *mex-1* by RNAi results in ectopic expression (arrows) of *tbx-35* in AB descendants, consistent with a transformation of the AB granddaughters to MS (Mello et al., 1992). (**D**) Expression of *tbx-35*::GFP (arrows) in early C descendants, consistent with a C to MS transformation in *pie-1(RNAi)* embryos (Mello et al., 1992). (**E**) Depletion of the divergent β-catenin *wrm-1* by RNAi results in an E to MS transformation (Rocheleau et al., 1997), and concomitant expression of *tbx-35*::GFP in both the E and MS lineages. (**F**) Although MS adopts an E-like fate in *pop-1(–)* embryos (Lin et al., 1995), expression of *tbx-35*::GFP occurred in the early MS lineage of all mutant embryos examined (*n*>30). (**G**) *tbx-35* mRNA accumulates in MS as detected by in situ hybridization. Seventy-eight percent of embryos at this stage (*n*=50) showed MS expression and 22% of embryos did not stain. (**H**) Ectopic *tbx-35* mRNA in a *mex-1(RNAi)* embryo. Seventy percent of embryos at this stage (*n*=54) showed ectopic expression, 9% showed normal MS-specific expression and 20% did not stain. (**I**) Ectopic activation of *tbx-35* in E in a *lit-1(t1512)* embryo grown at 25°C (Kaletta et al., 1997). Sixty-five percent of embryos (*n*=55) showed expression in MS and E, 22% showed MS-specific expression and 13% did not stain. (**J**) Normal expression of *tbx-35* mRNA in a *pop-1(RNAi)* embryo. Seventy-eight percent of embryos (*n*=65) showed staining in MS, while 22% did not stain. For these and subsequent embryo images, anterior is towards the left, and dorsal is upwards, and the eggshell (seen by Nomarski optics) is shown with a broken blue line. Scale bar: 10 μm.

a 190 bp fragment of the *tbx-35* promoter (Fig. 2C). Competition with an oligonucleotide segment of the *end-1* promoter containing a single MED site abolished this shift, whereas a competitor with a mutated MED site competed only weakly. As MED-1 and MED-2 are 98% identical and functionally redundant, as evidenced by the ability of either *med-1* or *med-2* to rescue the lethality of *med-1,2(–)* embryos (Coroian et al., 2005), we conclude that both MED-1 and MED-2 activate *tbx-35*.

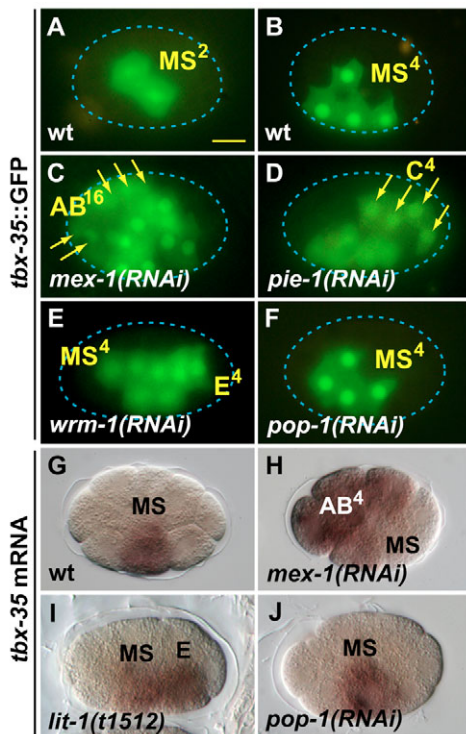### *tbx-35* is expressed in cells that have MS identity

As embryonic activation of *tbx-35* is restricted to MS, we assessed association of *tbx-35* with 'MS fate' by evaluating its expression in mutant backgrounds that generate ectopic MS-like cells. A *tbx-35*::GFP reporter is activated ectopically in the AB lineage in *mex-1(RNAi)* embryos and in the C lineage in *pie-1(RNAi)* embryos (Fig. 3C,D), consistent with the position of ectopic MS-like cells made in these mutants (Mello et al., 1992). We confirmed by in situ hybridization that *tbx-35* mRNAs are found in the AB lineage in *mex-1(RNAi)* embryos (Fig. 3H). Depletion of the β-catenin WRM-1 results in the ectopic activation of *tbx-35*::GFP in the early E lineage (Fig. 3E), as anticipated by the E to MS transformation in such animals (Rocheleau et al., 1997). Finally, *tbx-35* is activated in both E and MS in embryos mutant for *lit-1* (Fig. 3I), which encodes a Nemo-like kinase required to transduce the Wnt/MAPK signal that distinguishes E from MS (Rocheleau et al., 1999). We conclude that *tbx-35* expression is associated with cells that adopt the MS fate.

### Wnt/MAPK-dependent restriction of *tbx-35* to MS does not require POP-1

As the Wnt/MAPK effector TCF/POP-1 is an activator in E, and a repressor in MS, it is not known how Wnt/MAPK signaling might direct an inverse pattern for *tbx-35* regulation, namely repression in E and activation in MS. Indeed, in *pop-1(RNAi)* and *pop-1(zu189)* mutants, *tbx-35*::GFP persisted in the MS lineage alone (Fig. 3F; data not shown). By in situ hybridization, we observed the same MS-specific activation of endogenous *tbx-35* in *pop-1(RNAi)* embryos (Fig. 3J). We conclude that nuclear differences between MS and E exist in the absence of *pop-1*, showing that POP-1 is not required for at least one property of the MS cell (activation of *tbx-35*). We further conclude that an uncharacterized Wnt/MAPK-dependent mechanism must exist to repress *tbx-35* in E (see Discussion).

### *tbx-35* is an essential gene

The foregoing studies establish that *tbx-35* is activated at the correct time and place to specify MS downstream of MED-1,2. To assess the developmental requirement for *tbx-35*, we attempted to obtain *tbx-35(RNAi)* embryos by gonadal injection of *tbx-35* dsRNA (Fire et al., 1998). Although 78% (107/138) of *tbx-35(RNAi)* embryos were viable, 22% (31/138) underwent developmental arrest. The majority had fewer than 100 cells, apparently caused by a non-specific effect (see Materials and methods), but three embryos completed morphogenesis and lacked the MS-derived region of the pharynx (not shown). As this proportion of mutants is impractically small (2% of total), we were fortunate to obtain two *tbx-35* chromosomal mutations (*tm618* and *tm1789*) from the laboratory of Shohei Mitani (Tokyo, Japan). The *tm618* allele is a 1276 bp deletion/13 bp insertion that removes 59 C-terminal codons and the putative 3′UTR (Fig. 2A). *tm1789* is a 922 bp deletion that removes much of the 5′ flanking region, including five out of seven MED-1 binding sites, and 91 N-terminal codons that include one-third of the conserved T-box domain. Hence, *tm1789* may be a null mutant.

Indeed, although *tm618* homozygotes are viable and demonstrate no obvious phenotype, *tm1789* embryos either fail to hatch or hatch as inviable larvae.

To test whether the lethality of *tm1789* results from loss of *tbx-35* function, we constructed a homozygous *tm1789* strain balanced with an extrachromosomal array consisting of cosmid ZK177 and an *unc-119*::CFP reporter (Maduro and Pilgrim, 1995). We obtained a line, MS516, in which all viable animals exhibit *unc-119*::CFP expression, and which segregates ~40% inviable animals that lack reporter expression, confirming rescue by ZK177. We were further able to rescue with a 2.4 kbp PCR product containing *tbx-35* with

734 bp of 5′ flanking sequence and 374 bp downstream of the predicted polyA site, allowing us to conclude that the lethality of *tm1789* results from loss of *tbx-35* function (see Materials and methods).

## *tbx-35(tm1789)* embryos specifically lack MS-derived tissues

The vast majority of *tm1789* homozygotes (>95%), hereafter referred to as '*tbx-35(–)*', die as embryos. Although wild-type embryos elongate to greater than 3× the length of the eggshell before hatching (Fig. 4A), ~25% of *tbx-35(–)* embryos elongate to only 1.5-fold (Fig. 4D), 60% elongate to twofold and 15% elongate to threefold. The least-elongated embryos are similar to *med-1,2(–)* embryos in three respects: *med-1,2(–)* embryos arrest at between onefold and twofold; approximately one-third of *med-1,2(–)* and *tbx-35(–)* embryos contain internal cavities, similar to the hypodermis-lined cavities seen in *skn-1(–)* embryos; and the pharynx is abnormally small and lacks a grinder, a marker of MS-derived pharynx (Bowerman et al., 1992; Maduro et al., 2001). However, although some *med-1,2(–)* embryos lack endoderm (Coroian et al., 2005; Goszczynski and McGhee, 2005; Maduro et al., 2001), all *tbx-35(–)* homozygotes make endoderm (not shown).

We examined pharynx production in *tbx-35(–)* embryos using a *ceh-22* reporter to mark pharynx muscle (Table 1) (Okkema and Fire, 1994). Although wild-type embryos display a normal pharynx with ABa-derived and MS-derived regions, *tbx-35(–)* embryos appear to lack the posterior, MS-derived region (Fig. 4B,E). Indeed, in situ hybridization of the pharynx-specifying gene *pha-4* and the pharyngeal myosin gene *myo-2* revealed smaller, anterior-specific domains of expression in *tbx-35(–)* embryos when compared with wild type (Fig. 5A,B,E,F) (Gaudet and Mango, 2002; Okkema et al., 1993). Production of ABa-derived pharynx requires a Notch/GLP-1-mediated cell-cell interaction between MS and the AB lineage (Priess et al., 1987). To determine if the remaining pharynx in *tbx-35(–)* embryos is ABa derived, we combined the *glp-1(or178)* mutation with *tbx-35(tm1789)*. Although *tbx-35(+); glp-1(–)* embryos contained an average of 5.7±0.2 (*n*=74) pharynx muscle cells (Fig. 4C), *tbx-35(–); glp-1(–)* embryos had a mean of 0.3±0.1 (*n*=74) (Fig. 4F). Therefore, loss of *tbx-35* greatly reduces, but does not completely eliminate, MS-derived pharynx. The slightly leaky nature of these *tbx-35(–)* defects contrasts with *med-1,2(–)* embryos, which never elongate past twofold and contain no MS-derived pharynx (Maduro et al., 2001).

We next assessed whether production of body muscles is altered in *tbx-35(–)* embryos using a MyoD/*hlh-1*::GFP reporter (Krause et al., 1994). At ~1.5-fold elongation, MS-derived muscles are found at the anterior end of the embryo (arrowheads in Fig. 4H) (Sulston et al., 1983). Similarly staged *tbx-35(–)* embryos lack these muscles, and instead show an accumulation of extra muscle cells in the middle of the embryo (Fig. 4K). The same pattern is observed with expression of the body muscle myosin gene *myo-3* by in situ hybridization (Fig. 5C,G). The occurrence of an apparent ectopic region of muscle cells suggests that MS still makes muscle in *tbx-35(–)* mutants. To test whether these are 'MS type' or 'C type' muscle cells, we depleted Caudal/PAL-1 by RNAi to specifically block formation of C- and D-type muscle (Hunter and Kenyon, 1996). Although *pal-1(RNAi)* blocks formation of C- and D-derived muscle, showing a lack of *hlh-1* expression where these muscles should be (arrows in Fig. 4I), nearly all muscle is absent in *tbx-35(–); pal-1(RNAi)* embryos (Fig. 4L). As MS-derived pharynx and body muscle are greatly reduced in *tbx-35(–)* embryos, we conclude that *tbx-35* is required to make the majority of the descendants of MS.
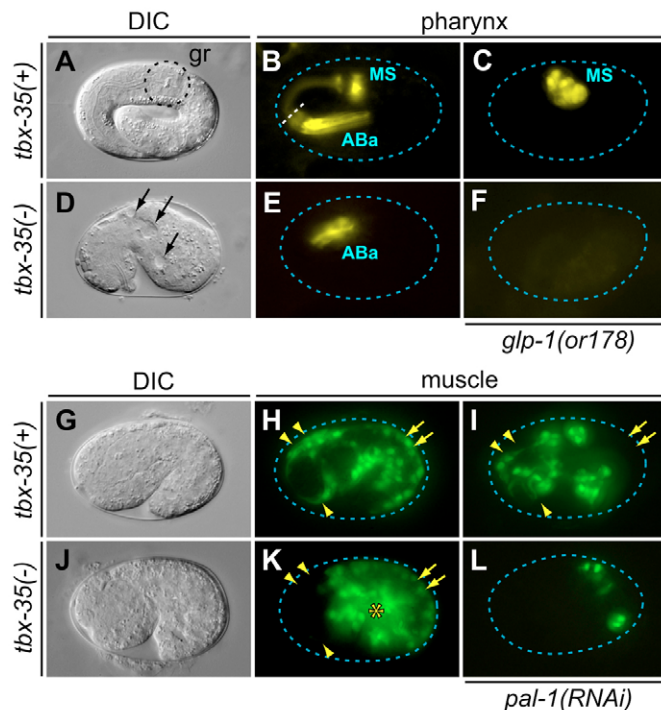


**Fig. 4. Deficiency of MS-derived cell types in *tbx-35(tm1789)* embryos.** (**A**) Differential interference contrast (DIC) image showing the grinder (gr), indicative of MS-derived pharynx, in a wild-type three-fold stage embryo. (**B**) A *ceh-22*::GFP reporter (pseudocolored yellow) shows the fully elongated pharynx with MS- and ABa-derived halves (Okkema and Fire, 1994). (**C**) Terminal *glp-1(or178)* embryo showing *ceh-22* expression in only MS-derived pharynx. (**D**) Terminal *tbx-35(tm1789)* embryo arrested at 1.5-fold elongation, similar in appearance to *med-1,2(–)* embryos (Coroian et al., 2005). The grinder is absent, and internal cavities (arrows) are observed in 35% of such embryos, similar to the hypodermis-lined voids in *skn-1(–)* embryos (Bowerman et al., 1992). (**E**) *ceh-22* expression in only ABa-derived pharynx in *tbx-35(–)*. (**F**) Absence of pharynx in a *glp-1(–); tbx-35(–)* embryo. (**G**) Wild-type embryo at the 1.5-fold stage. (**H**) Expression of *hlh-1*::GFP in the embryo shown in G indicates muscle precursors (Chen et al., 1994). In wild type, an average of 46.8±1.4 cells (*n*=10) were scored as expressing at this stage. (**I**) In terminal *pal-1(RNAi)* embryos, muscles made by C and D are not made, resulting in a loss of posterior *hlh-1* expression (arrows; compare arrows in H). Expression was seen in an average of 20±0.6 cells (*n*=33). (**J**) A 1.5-fold stage *tbx-35(tm1789)* embryo. (**K**) *hlh-1*::GFP expression in the embryo shown in J shows lack of signal in the anterior (arrowheads; compare H), while posterior expression persists (arrows) and a large cluster of *hlh-1*-expressing cells is seen near the center (*). An average of 34.8±2.4 cells (*n*=10) was scored in these embryos. (**L**) Terminal *tbx-35(–); pal-1(RNAi)* embryos express *hlh-1*::GFP in only a small number of cells, with an average of 5.7±0.5 cells (*n*=40).

**Table 1. Cell types made in mutant embryos**

| Genotype | Number of pharynx muscle cells (ceh-22::GFP) | Number of body muscle cells (hlh-1::GFP) | Number of hypodermal cells (lin-26::GFP) |
|---|---|---|---|
| tbx-35(tm1789); Ex[tbx-35(+)] | 13.1±0.2 (84) | 46.8±1.4 (10) | nd |
| tbx-35(tm1789) | 4.7±0.2 (68)* | 34.8±2.4 (10)* | nd |
| glp-1(or178); tbx-35(tm1789); Ex[tbx-35(+)] at 25°C | 5.7±0.2 (74) | nd | nd |
| glp-1(or178); tbx-35(tm1789) at 25°C | 0.3±0.1 (74)* | nd | nd |
| glp-1(or178); wrm-1(RNAi); tbx-35(tm1789); Ex[tbx-35(+)] at 25°C | 22.6±0.7 (42) | nd | nd |
| glp-1(or178); wrm-1(RNAi); tbx-35(tm1789) at 25°C | 5.6±0.7 (49)* | nd | nd |
| mex-1(RNAi); pie-1(RNAi); tbx-35(tm1789); Ex[tbx-35(+)] | 30.0±1.8 (26) | >25 (54)[†] | 6.5±2.1 (16) |
| mex-1(RNAi); pie-1(RNAi); tbx-35(tm1789) | 1.0±0.2 (38)* | >25 (41)[†] | 26.7±3.9 (11)* |
| pal-1(RNAi); tbx-35(tm1789); Ex[tbx-35(+)] | nd | 20.2±0.6 (33) | nd |
| pal-1(RNAi); tbx-35(tm1789) | nd | 5.7±0.5 (40)* | nd |
| pie-1(RNAi); tbx-35(tm1789); Ex[tbx-35(+)] | nd | 30.4±0.8 (36) | nd |
| pie-1(RNAi); tbx-35(tm1789) | nd | 20.8±1.3 (34)* | nd |
| pie-1(RNAi); pal-1(RNAi); tbx-35(tm1789); Ex[tbx-35(+)] | nd | 29.4±0.9 (25) | nd |
| pie-1(RNAi); pal-1(RNAi); tbx-35(tm1789) | nd | 4.4±0.6 (25)* | nd |
| mex-1(RNAi); pie-1(RNAi); pal-1(RNAi); tbx-35(tm1789); Ex[tbx-35(+)] | nd | >25 (30)[†] | 1.7±0.4 (31) |
| mex-1(RNAi); pie-1(RNAi); pal-1(RNAi); tbx-35(tm1789) | nd | 4.9±0.8 (19)* | 12.5±3.5 (13)* |

*Significant difference (P<0.01) compared with the experiment on the line immediately above.
[†]All embryos had at least 25 GFP-expressing cells, but the total number per embryo was not counted because of the difficulty of resolving individual cells in these backgrounds.
nd, not determined.

The production of ectopic C-type muscle in *tbx-35(–)* mutants is anticipated from the observation that both *skn-1(–)* and *med-1,2(–)* embryos display a transformation of MS into a C-like cell (Bowerman et al., 1992; Hunter and Kenyon, 1996; Maduro et al., 2001). We examined early *tbx-35(–)* embryos for evidence of ectopic activation of zygotic *pal-1*, a marker for the early C lineage (Baugh et al., 2005). By in situ hybridization, we observed zygotic *pal-1* transcripts in only the early C lineage in wild-type (Fig. 5D) and in both the C and MS lineages in ~30% of *tbx-35(–)* embryos (Fig. 5H). We observed similar ectopic expression in *tbx-35(–); pal-1*::YFP embryos (data not shown). With both approaches, ectopic *pal-1* signal in the MS lineage occurred slightly later than that of the C lineage, and with variable intensity. We conclude that the MS blastomere in *tbx-35(–)* embryos exhibits a transformation of MS to C of varying expressivity.
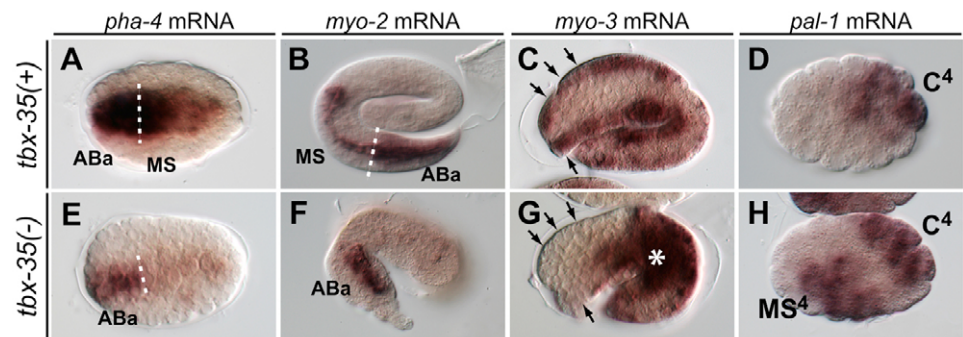
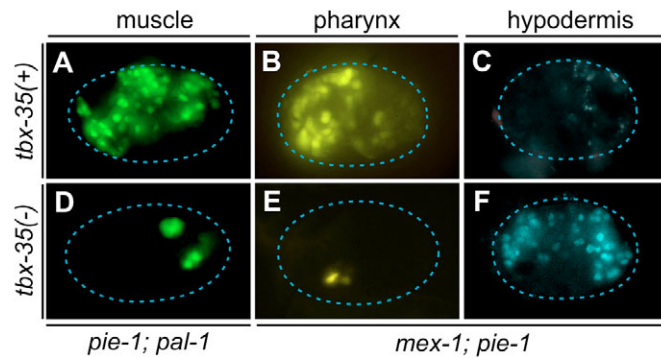### *tbx-35* is required for ectopic MS-like fates

The results thus far suggest *tbx-35* plays an important role in MS specification. We next evaluated the requirement for *tbx-35* in specification of abnormal MS-like cells that arise in specific mutant backgrounds. In *pie-1(–)* embryos, C adopts an MS-like fate, resulting in embryos in which nearly all muscle cells are derived from MS (Mello et al., 1992). As loss of *tbx-35* leads to apparent production of C-type muscle from MS, we simultaneously depleted *pie-1* and *pal-1* by RNAi to ensure the generation of only MS-type muscle in these embryos. We found that although *tbx-35(+); pie-1(RNAi); pal-1(RNAi)* embryos generated an average of 29.4±0.9 (*n*=25) muscle cells, *tbx-35(–); pie-1(RNAi); pal-1(RNAi)* embryos made only 4.4±0.6 (*n*=25) muscle cells (Fig. 6A,D), consistent with a requirement for *tbx-35* function in the mis-specification of the C cell as 'MS' in *pie-1* mutants.



**Fig. 5. MS lineage defects in *tbx-35(–)* embryos confirmed by in situ hybridization.** (**A**) *pha-4* transcripts in a wild-type onefold embryo have an intense pharynx component (with ABa- and MS-derived regions) and a weaker endoderm-specific component. All stained embryos (*n*=37) demonstrated this type of staining. (**B**) Expression of the pharyngeal myosin gene *myo-2* in a wild-type late-stage embryo. All (*n*=37) stained embryos displayed a pattern similar to the one shown. (**C**) Expression of the body muscle myosin gene *myo-3* mRNA in a twofold embryo. Anterior muscles (descendants of MS) are shown by arrows. (**D**) Zygotic activation of *pal-1* in the early C lineage. Among 31 embryos at this stage, four showed no staining, while the remaining 27 (87%) showed expression only in the C lineage, as shown here. (**E**) Eighty-eight percent (*n*=25) of stained *tbx-35(–)* embryos displayed a reduced anterior region of high *pha-4* expression, similar to the embryo shown here. Twelve percent of embryos displayed apparent wild-type staining (not shown). (**F**) Detection of *myo-2* in only the anterior half of the pharynx in *tbx-35(–)*. (**G**) Absence of anterior MS-derived muscle (arrows) in a *tbx-35(–)* embryo stained for *myo-3*. Additional staining is present in the center of the embryo (*). (**H**) Detection of zygotic *pal-1* mRNA in both the MS and C lineages in a *tbx-35(–)* embryo, evidence of an early MS to C transformation. Among 36 embryos from the MS516 strain, three showed no staining and 33 showed strong expression in the C lineage. Of these, six also showed strong signal in the MS lineage, and two showed weak MS signal. As the rescuing array in MS516 is inherited by 60% of embryos (*n*=94), we estimate that ~30% of *tbx-35(–)* embryos express *pal-1* in the MS lineage.

**Fig. 6. *tbx-35* is required to specify ectopic MS-like cells.**
(**A**) Ectopic MS-type muscle (*hlh-1*::GFP) in *tbx-35(+); pie-1(RNAi); pal-1(RNAi)* embryos. (**B**) Ectopic pharynx cells (*ceh-22*::GFP, pseudocolored yellow) in a *tbx-35(+); mex-1(RNAi); pie-1(RNAi)* embryo. (**C**) Very few hypodermal cells (*lin-26*::GFP, pseudocolored cyan) are made in a *tbx-35(+); mex-1(RNAi); pie-1(RNAi)* embryo (an average of 6.5±2.1 cells, *n*=16). (**D**) Dramatic reduction in number of muscle cells in a *tbx-35(–); pie-1(RNAi); pal-1(RNAi)* embryo. (**E**) Near absence of pharynx in a *tbx-35(–); mex-1(RNAi); pie-1(RNAi)* embryo. (**F**) Production of hypodermal cells in a *tbx-35(–); mex-1(RNAi); pie-1(RNAi)* embryo (an average of 26.7±3.9 nuclei, *n*=11). Simultaneous depletion of *pal-1* greatly reduces the production of these extra hypodermal cells (Table 1).

We next examined production of ectopic MS-type tissues in *mex-1(–); pie-1(–)* embryos. In such mutants, the AB grand-daughters and C adopt MS-like fates, leading to production of six MS-like cells overall (Fig. 1), while eliminating sources of non-MS-type muscle and pharynx (Mello et al., 1992). Large numbers of pharynx cells (30.0±1.8, *n*=26) were seen in *tbx-35(+); mex-1(RNAi); pie-1(RNAi)* embryos (Fig. 6B), which were nearly eliminated (1.0±0.2 cells, *n*=38) when *tbx-35* was mutant (Fig. 6E). Production of MS-type muscle in a *mex-1(–); pie-1(–)* background was also found to be strongly dependent on *tbx-35* (Table 1). We further found that the number of cells expressing the hypodermal marker *lin-26* (Labouesse et al., 1996) was increased in a *tbx-35(–); mex-1(–); pie-1(–)* background over *tbx-35(+); mex-1(–); pie-1(–)* (Fig. 6C,F). As C makes hypodermal cells, this result is consistent the transformation of at least some MS-like cells to C-like cells in the absence of *tbx-35*.

Last, we examined production of pharynx in a *wrm-1(RNAi)* background, in which E adopts an MS-like fate (Rocheleau et al., 1997). To block formation of AB-derived pharynx, we used a *glp-1(or178)* mutation. We found that although *tbx-35(+); glp-1(or178); wrm-1(RNAi)* embryos make 22.6±0.7 pharynx cells (*n*=42), *tbx-35(–); glp-1(or178); wrm-1(RNAi)* embryos make only 5.6±0.7 cells (*n*=49). We conclude that *tbx-35* is crucial for specification of MS fate in both its normal context and in genetic backgrounds that result in production of ectopic MS-like cells.

## *tbx-35* overexpression is sufficient to specify MS-like fates

The expression of *tbx-35* in MS-like precursors, and the requirement for *tbx-35* to generate MS-derived fates, suggest that TBX-35 alone might be sufficient to specify the MS fate. As some Tbx proteins function as classical activators (Naiche et al., 2005), we tested whether TBX-35 can reprogram non-MS cells to become mesoderm precursors, similar to the ability of MED-1 to promote the generation of ectopic pharynx and endoderm (Maduro et al., 2001). A heat shock (hs) *tbx-35* transgene was constructed and introduced
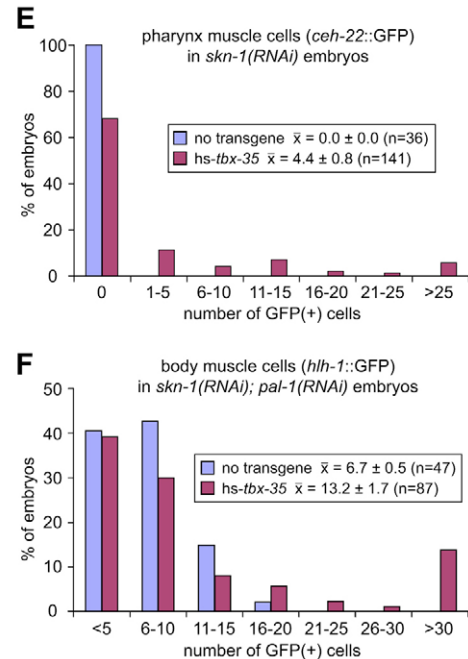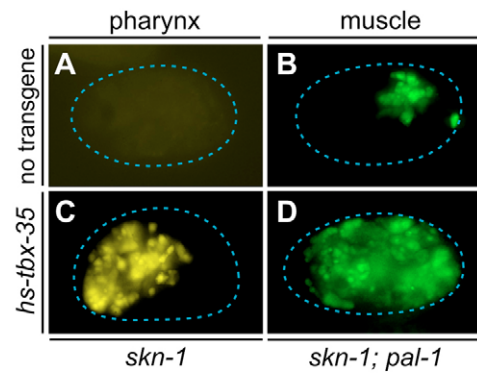


**Fig. 7. Overexpression of TBX-35 is sufficient to specify pharynx and muscle fates.** Images depict terminal embryos that received a 30-minute heat shock at 33°C before the 100-cell stage. (**A**) Absence of pharynx muscle (*ceh-22*::GFP) in a *skn-1(RNAi)* embryo (0.0±0.0 cells, *n*=36). (**B**) Small numbers of muscle cells (*hlh-1*::GFP) made in a *skn-1(RNAi); pal-1(RNAi)* background (6.7±0.5 cells, *n*=47). (**C**) Pharynx muscle cells are made when *tbx-35* is overexpressed in a *skn-1(RNAi)* background. Thirty-two percent of embryos (45/141) had an average of 13.8±1.8 cells per embryo, with 10 embryos containing at least 25 cells as shown here, or an overall average of 4.4±0.8 (*n*=141). (**D**) An increase in body muscle cells in a *skn-1(RNAi); pal-1(RNAi)* background following ectopic activation of *tbx-35* (an average of 13.2±1.7 cells, *n*=87). Fifteen percent of embryos (13/87) had at least 30 muscle cells, similar the embryo shown here. (**E**) Numbers of pharynx muscle cells (*ceh-22*::GFP) in a *skn-1(RNAi)* background. (**F**) Numbers of body muscle cells (*hlh-1*::GFP) in a *skn-1(RNAi); pal-1(RNAi)* background.

into the *ceh-22* and *hlh-1* reporter strains. We used *skn-1(RNAi)* and *skn-1(RNAi); pal-1(RNAi)* backgrounds to block specification of pharynx and muscle precursors, respectively (Bowerman et al., 1992; Hunter and Kenyon, 1996). As shown in Fig. 7, expression of hs-*tbx-35* can promote development of pharynx and muscle in a significant proportion of embryos (30% in the case of pharynx). Heat shock of *skn-1(RNAi)* embryos carrying a hs-*tbx-35*[E42V] transgene, containing a missense change in a conserved amino acid (Fig. 2B), resulted in embryonic arrest without production of
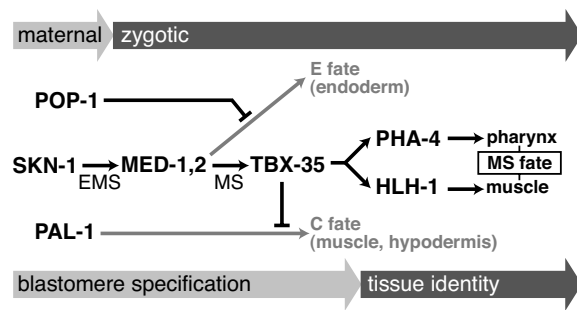
**Fig. 8. A model for specification of the MS blastomere.** A gene cascade that begins with maternal SKN-1 progresses through activation of *med-1,2* in EMS, which in turn activate *tbx-35* in MS. POP-1 represses endoderm specification in MS. In E, the sister of MS, a Wnt/MAPK-dependent mechanism represses *tbx-35* through an unknown effector (not shown). TBX-35 acts upstream of genes that specify pharynx and muscle fates, through regulators such as *pha-4* and *hlh-1*, respectively (Gaudet and Mango, 2002; Krause et al., 1990). TBX-35 also represses targets of maternal PAL-1, blocking C fates. Activation of *med-1,2* by SKN-1 is direct (Maduro et al., 2001), as is activation of *tbx-35* by MED-1,2 (this work). In addition to *med-1,2*, SKN-1 also activates the expression of one or more Delta/Serrate/Lag (DSL) proteins that enable the MS cell to signal the AB lineage to make anterior pharynx (Priess et al., 1987).

pharynx (*n*>50). We conclude that high levels of TBX-35 are sufficient to specify MS fates. As this activity requires a conserved amino acid in its predicted DNA-binding domain, TBX-35 appears to function as a classical transcriptional activator.

## DISCUSSION

We have shown that the T-box gene *tbx-35* specifies the fate of the *C. elegans* MS blastomere. MED-1 directly activates *tbx-35* in MS, and a Wnt-dependent POP-1-independent mechanism blocks this activation in the E cell; loss of *tbx-35* function results in a drastic reduction in MS-derived cell types, both in the context of a normal MS blastomere and in mutants that make additional MS-like cells; and overexpression of *tbx-35* is sufficient to specify MS fates. As shown in Fig. 8, our data place *tbx-35* in the pathway for MS specification immediately downstream of *med-1,2* and upstream of tissue-specific regulators such as the pharynx identity gene FoxA/*pha-4* and the muscle identity gene MyoD/*hlh-1* (Fukushige and Krause, 2005; Gaudet and Mango, 2002).

### TBX-35-independent MS fates

Our results suggest that *skn-1(–)* and *med-1,2(–)* embryos fail to make MS-derived cell types because they do not activate *tbx-35*. Although loss of *med-1,2* leads to an almost complete loss of MS-derived tissues and embryonic lethality, even in embryos that make a gut (Bowerman et al., 1992; Maduro et al., 2001), some *tbx-35(tm1789)* mutants elongate to threefold and even hatch before arresting. As no *tbx-35(–)* embryos escape lethality, there is probably no redundant activity that can completely compensate for the absence of TBX-35 function.

Do additional regulators specify at least some MS-like fates? Our observation that MS lineage activation of zygotic *pal-1* in *tbx-35(–)* embryos is of variable intensity is consistent with the existence of other MS fate-promoting, C-repressing regulators. At least two more genes, *hlh-25* and *hlh-27*, encoding homologs of the bHLH family of transcription factors, are activated in MS at the same time as *tbx-35* (Broitman-Maduro et al., 2005).

Overexpression of *hlh-25* can specify some early cells as muscle progenitors, suggesting that HLH-25/27 may participate directly in muscle specification, or indirectly in other aspects of muscle inductions known to involve MS (Broitman-Maduro et al., 2005; Schnabel, 1995). Alternatively, targets of TBX-35 may be able to respond directly to residual MED-1,2, or other activators, present in the early MS lineage.

### Mesoderm versus endoderm: a simpler network for a more complex lineage?

The embryonic MS and E lineages are remarkably different. The E lineage generates 20 cells of a single type (gut), while MS generates 80 cells of various types, including pharynx, muscle, coelomocytes and cells of the somatic gonad (Sulston et al., 1983). E specification requires an extrinsic induction, transduced through an overlapping Wnt/MAPK/Src pathway, while activity of TCF/POP-1, Caudal/PAL-1, SKN-1 and MED-1,2 all contribute to E specification (Bei et al., 2002; Goldstein, 1992; Maduro et al., 2005b; Rocheleau et al., 1997; Shetty et al., 2005; Thorpe et al., 1997). By contrast, MS identity is an intrinsic property of an EMS daughter (Goldstein, 1992), and our data show that MS may be specified by the relatively simple linear pathway, SKN-1→MED-1,2→TBX-35.

Although specification of the MS cell appears to be comparatively simple, the development of its descendants requires the deployment of multiple gene batteries. For one, a complex gene network is deployed by the regulator FoxA/PHA-4 to specify the developing pharynx, an organ that itself is composed of different cell types such as neurons and muscle (Gaudet and Mango, 2002; Sulston et al., 1983). As MS-derived pharynx cells arise from only two of the four granddaughters of MS (MSaa and MSpa), additional factors must restrict pharynx fate to these sublineages (Sulston et al., 1983). As TCF/POP-1 displays nuclear anteroposterior differences in these cells (Lin et al., 1998; Maduro et al., 2002), the Wnt/MAPK pathway is likely to be involved in differential activation of pharynx potential within the MS lineages. The most obvious conclusion from the differences between MS and E, then, is that the complexity of the lineage elaborated by an early embryonic cell is not necessarily correlated with the nature of the gene networks that specify the progenitor itself.

### Wnt-dependent repression of mesoderm

We have found that *tbx-35* is repressed in E in a Wnt/MAPK-dependent manner, as depletion of β-catenin/WRM-1 or Nemo/LIT-1 results in ectopic activation of *tbx-35* in E. In the absence of *pop-1*, activation of *tbx-35* persists in the MS cell, even though the endoderm genes *end-1* and *end-3* become activated in both MS and E (Maduro et al., 2005a). The persistence of *tbx-35* expression in MS in *pop-1(–)* embryos, and the E to C transformation in *end-1,3(–)* embryos, suggest that repression of *tbx-35* in E is not achieved by END-1,3. As MS (like E) adopts an endoderm fate in *pop-1(–)* embryos, END-1,3 outcompete TBX-35 when both are present together in MS. Hence, depletion of a Wnt-dependent repressor of *tbx-35* might not demonstrate loss of endoderm. In *Xenopus*, HMG proteins of the Sox family can directly interact with β-catenin, blocking activation of Wnt target genes by depleting this necessary TCF co-activator (Zorn et al., 1999), although in the case of the MS-E decision, such a mechanism would probably block endoderm specification itself. The *C. elegans* genome encodes at least three Sox-like genes (indexed in WormBase, release WS156), one of which (*sox-1*) we have shown to be expressed in the early MS and E lineages (Broitman-Maduro et al., 2005); however, the role of any

of these in MS or E specification is not yet known, and as *C. elegans* continually surprises the development field with unexpected Wnt/MAPK features (Kidd, 3rd et al., 2005), we can make no reliable predictions. The identification of such an effector would provide an additional mechanism by which Wnt/MAPK-dependent nuclear differences could be established in *C. elegans* development (Kaletta et al., 1997; Rocheleau et al., 1999).

## T-box genes in mesoderm development

The *C. elegans* pharynx appears to be an organ whose development requires the activity of multiple Tbx factors in different lineages. Although *tbx-35* specifies MS, the redundant genes *tbx-37* and *tbx-38* are required for the GLP-1-mediated induction that specifies the ABa-derived precursors of the largely anterior half of the pharynx (Good et al., 2004). More recently, yet another Tbx gene, *tbx-2*, was found to be required for development of ABa-derived pharynx muscles (Chowdhuri et al., 2006).

Tbx regulators are essential for heart development in vertebrates; in humans, mutations in Tbx genes lead to congenital cardiac defects (Plageman and Yutzey, 2005). Recently, Tbx genes have been found to be important for development of heart in *Drosophila*, in which the Tbx genes *Dorsocross*, *midline* and *H15* function in the specification and formation of cardiac cells (Miskolczi-McCallum et al., 2005; Reim and Frasch, 2005). Although *C. elegans* lacks a circulatory system, its pharynx has some similarities to a heart, as it is a pumping organ containing contractile muscle that is distinct from body muscle (Avery and Shtonda, 2003; Okkema et al., 1993). Indeed, pharynx developmental defects in *C. elegans ceh-22* mutants can be rescued by expression of its vertebrate homolog, the heart specification gene *Nkx2.5* (Haun et al., 1998). As MS descendants produce most of the posterior pharynx (and MS induces the specification of the remainder from the AB lineage), is the specification of MS by a Tbx factor truly an example of homology? Primitive bilaterians are thought to have evolved regions of contractile mesoderm that functioned as a primordial heart (Bishopric, 2005). Specification of cells within such a tissue territory may have been controlled directly by Tbx regulators. Extant organisms such as *C. elegans*, in which cell fates are specified early, might possess Tbx gene cascades into which other layers of regulation have been intercalated (Erwin and Davidson, 2002). Hence, the use of *tbx-35* to specify MS may reflect its derivation from a more direct role for Tbx proteins in direct activation of genes in differentiated mesoderm.

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