The myogenic potency of HLH-1 reveals wide-spread developmental plasticity in early *C. elegans* embryos

Tetsunari Fukushige and Michael Krause*

Laboratory of Molecular Biology, National Institute of Diabetes, Digestive, and Kidney Diseases, NIH, Bethesda, MD 20892, USA
*Author for correspondence (e-mail: mwkrause@helix.nih.gov)

Accepted 10 February 2005

Development 132, 1795-1805
Published by The Company of Biologists 2005
doi:10.1242/dev.01774

Summary

In vertebrates, striated muscle development depends on both the expression of members of the myogenic regulatory factor family (MRFs) and on extrinsic cellular cues, including Wnt signaling. The 81 embryonically born body wall muscle cells in *C. elegans* are comparable to the striated muscle of vertebrates. These muscle cells all express the gene *hlh-1*, encoding HLH-1 (CeMyoD) which is the only MRF-related factor in the nematode. However, genetic studies have shown that body wall muscle development occurs in the absence of HLH-1 activity, making the role of this factor in nematode myogenesis unclear. By ectopically expressing *hlh-1* in early blastomeres of the *C. elegans* embryo, we show that CeMyoD is a bona fide MRF that can convert almost all cells to a muscle-like fate, regardless of their lineage of origin. The window during which ectopic HLH-1 can function is surprisingly broad, spanning the first 3 hours of development when cell lineages are normally established and non-muscle cell fate markers begin to be expressed. We have begun to explore the maternal factors controlling zygotic *hlh-1* expression. We find that the Caudal-related homeobox factor PAL-1 can activate *hlh-1* in blastomeres that either lack POP-1/TCF or that have down-regulated POP-1/TCF in response to Wnt/MAP kinase signaling. The potent myogenic activity of HLH-1 highlights the remarkable developmental plasticity of early *C. elegans* blastomeres and reveals the evolutionary conservation of MyoD function.

Key words: *C. elegans*, MyoD, Muscle, Myogenesis

Introduction

The transcriptional regulation of vertebrate skeletal muscle development is well understood (reviewed by Buckingham, 2001; McKinsey et al., 2001; Pownall et al., 2002; Tajbakhsh, 2003; Buckingham et al., 2003). Sonic hedgehog and Wnt signals, from the notochord and neural tube respectively, work in concert with the homeobox gene *Pax-3* to turn on the early-acting myogenic regulatory factors (MRFs) *Myf-5* and *MyoD* in the adjacent somites. These two basic helix-loop-helix (bHLH) MRFs initiate a transcriptional cascade, including the activation of two closely related bHLH MRFs, *MRF-4* and *myogenin*, that culminates in the expression of terminal skeletal muscle gene products (e.g. myosin heavy chain, actin) needed for differentiation (McKinsey et al., 2001; Berkes et al., 2004). These events are integrated with growth signals so that proliferative myoblasts exit the cell cycle at the appropriate point in development (reviewed by Sabourin and Rudniki, 2000; Wei and Paterson, 2001).

In *C. elegans*, the striated body wall muscle cells are comparable to vertebrate skeletal muscle. This musculature provides the locomotive force for the animal and consists of 95 mononucleated cells, arranged in four quadrants along the length of the body (Waterston, 1988). Eighty-one of these cells are born and differentiate during embryogenesis (Sulston et al., 1983), while 14 more are added post-embryonically (Sulston and Horvitz, 1977). Although previous studies have identified several factors required for post-embryonic muscle development (Harfe et al., 1998a; Harfe et al., 1998b; Liu and Fire, 2000; Corsi et al., 2000), there has been little progress in understanding embryonic striated myogenesis. One general conclusion of these studies is that embryonic and post-embryonic muscle development are controlled by different sets of transcription factors, an unexpected finding given that muscle cells born during these two different periods of development appear morphologically and functionally equivalent.

In *C. elegans*, fertilization is followed by a rapid set of embryonic cellular divisions that generate five somatic founder blastomeres called AB, MS, E, C and D, and the germline blastomere P4. The 81 embryonic body wall muscles are derived from four of the five somatic founders AB (1), MS (28), C (32) and D (20). The D lineage gives rise exclusively to body wall muscle whereas the other lineages give rise to multiple cell fates. Maternal effect mutations have been identified that alter the fate of one body wall muscle-producing founder without affecting other lineages. For example, *skn-1* mutants lack the 28 MS-derived body wall muscles but body wall muscle from the C and D lineages are present (Bowerman et al., 1992; Bowerman, 1995; Bowerman et al., 1997). Conversely, *pal-1* mutants lack the C and D lineage-derived body wall muscle cells without affecting those derived from MS (Hunter and Kenyon, 1996; Ahringer, 1997). This founder blastomere autonomy, with respect to body wall muscle formation, demonstrates that there are several genetically distinct pathways for embryonic striated muscle development.

However, it is yet to be determined if these independent genetic pathways converge on a common molecular nodal point to regulate body wall muscle cell fate.

A single *C. elegans* gene, *hlh-1,* encodes a transcription factor (HLH-1, a.k.a. CeMyoD) that is related to the vertebrate *MRFs*. *hlh-1* is zygotically expressed in embryonic body wall muscle precursors and their differentiated descendants, beginning at the ~90 cell stage of development (Krause et al., 1990). This expression pattern suggested that *hlh-1* might represent a nodal point for body wall muscle development, analogous to the role of *Myf-5* and *MyoD* in vertebrate myogenesis. Homozygous *hlh-1* null mutant animals complete embryogenesis but are paralyzed upon hatching, have severe morphological defects, and usually die during the first larval stage, revealing an essential role for HLH-1 in muscle development and function (Chen et al., 1992; Chen et al., 1994). However, *hlh-1* null mutants have 81 embryonic body wall muscle cells and express many terminal muscle products at wild-type levels. Thus, HLH-1 is not essential for body wall muscle cell fate, and one or more additional factors must be involved in myogenic determination. In fact, the *hlh-1* knockout allele phenotypes challenge the notion that HLH-1 is itself myogenic, suggesting instead that it may be downstream of myogenic factors in the body wall muscle transcriptional cascade. Similar results in *Drosophila* for the MRF-related gene *nautilus* have suggested that there may be a fundamental difference between vertebrates and invertebrates with regard to the regulation of striated muscle development (Michelson et al., 1990; Balagopalan et al., 2001).

The present study further defines the function of HLH-1 and its relationship to intrinsic and extrinsic factors regulating early development. By ectopically activating HLH-1 in the early *C. elegans* embryo, we show that HLH-1 alone is sufficient to convert most cells of the early embryo into a body wall muscle-like fate. This is true for cells that would normally give rise to either ectoderm or endoderm, demonstrating that HLH-1 is a bona fide MRF with potent myogenic activity. The ectopic myogenic activity of HLH-1 is limited to undifferentiated blastomeres and spans several hours of early development, including times when non-muscle, lineage-restricted markers are normally expressed. We find that the Caudal-related factor PAL-1 can activate *hlh-1*, providing a link between maternal factors and HLH-1 activation, and we explore the role of Wnt/MAP kinase signaling in making cells competent for myogenesis. These studies demonstrate a level of developmental plasticity in *C. elegans* that had previously not been appreciated.

Materials and methods

Strains

The following strains of *Caenorhabditis elegans* were used: wild type (N2); heat-shock *hlh-1* (strains KM267, KM289), *hlh-1* null allele (*cc450*) in the *mc6*-balanced strain PD4849, *hlh-1* temperature-sensitive allele (*cc561*) strain PD4605, *hlh-1::gfp* strain PD7963 and *myo-3::gfp* strain PD4251, all provided by A. Fire (Stanford University School of Medicine, CA, USA), *elt-2::gfp* strain JM63 from J. McGhee (The University of Calgary, Alberta, Canada), and heat-shock *pal-1* strains JA1179 and JA1180 provided by J. Ahringer (University of Cambridge, UK).

The heat-shock *hlh-1* construct was made by transferring a fragment containing the full-length *hlh-1* cDNA (Krause et al., 1990) into the *hsp 16.41* (Stringham et al., 1992) vector pPD49.83 (kindly provided by A. Fire) to yield the plasmid pKM1211. Animals harboring pKM1211 were generated by standard techniques using 100 μg of pKM1211 and 50 μg of the selectable dominant *rol-6* plasmid pRF4 (Mello and Fire, 1995). Integrated heat-shock *hlh-1* lines were generated by gamma irradiation (Egan et al., 1995) of extrachromosomal transformants and were back-crossed twice with the wild-type strain (N2) prior to use.

The heat-shock experiments

One and two cell stage embryos were isolated from transgenic hermaphrodites containing heat-shock expression constructs or from N2 controls. Embryos were heat shocked immediately or incubated for various times at room temperature (~22°C) prior to heat shock. For all treatments, heat shock consisted of a single 30 minute pulse at 34°C. The heat-shocked embryos were examined over time for the expression of cell type-specific reporter genes or incubated and fixed for antibody staining with markers for muscle, gut, hypodermis and germline (see below). Using the absence of hypodermal marker staining as a sensitive assay for the degree of myogenic conversion, we determined that the optimal conditions for HLH-1 myogenic activity were to isolate one- to two-cell embryos and incubate for 60 minutes prior to heat shock. Heat shock induction of PAL-1 was carried out after 20 minutes incubation of isolated embryos.

Antibody staining

Embryos were fixed with 5% paraformaldehyde in phosphate-buffered saline (PBS) on ice for 15 minutes and transferred onto 0.1% gelatin-coated slides, placed on an aluminum block on dry ice, freeze-cracked, methanol fixed at –20°C for 6 minutes, and rehydrated in PBS at room temperature. The primary antibodies used were raised against myosin heavy chain A [MHC A (Miller et al., 1986)], HLH-1 (Krause et al., 1990), ELT-2 (Fukushige et al., 1998) (a gift from Jim McGhee), LIN-26 (Labouesse et al., 1996) (a gift from Michel Labouesse), a pharyngeal muscle epitope [3NB12 (Priess and Thomson, 1987)], UNC-89, UNC-98 (Benian et al., 1996; Mercer et al., 2003) (a gift from Guy Benian), PAT-3 (Gettner et al., 1995) (a gift from Don Moerman), and germline P-granules [OIC1D4 (Strome and Wood, 1983)]. Secondary antibodies were fluorescein- or rhodamine-conjugated donkey anti-rabbit or goat anti-mouse IgG (Jackson Immunological).

RNAi

Double-stranded RNA corresponding to the genes *mex-1*, *mex-3*, *lit-1*, *wrm-1*, *skn-1* and *pal-1* were amplified from cDNA clones that were generated by reverse transcriptase-polymerase chain reaction (RT-PCR) with the primers listed below. These partial cDNA PCR products were inserted into the vector L4440 (Timmons et al., 2001) and served as a template for in vitro transcription to produce double stranded RNA for injection. The *pop-1* RNAi plasmid RL499 was a gift from R. Lin.

Primers used for PCR amplification of cDNA clones for RNAi:
MEX-1F (24 bp) 5′-ATGCAATCTTCAAATGGAGAGCAT-3′,
MEX-1R (24 bp) 5′-TTATCTCGAATAATGATCTTCGTG-3′,
MEX-3F (33 bp) 5′-AAGGATCCATGAAGGAAGAACAA-ATCGCCTATA-3′,
MEX-3R (25 bp) 5′-TTGGCAGATCTTGTTTCGCCGATTG-3′.
LIT-1F (24 bp) 5′-CATCCGGCCGCCGTCGGCTCTACG-3′,
LIT-1R (24 bp) 5′-CGGAGCACATGGTTCTTGGCTCCC-3′.
WRM-1F (23 bp) 5′-ATGGATGTGGATTGCGCAGAAAC-3′,
WRM-1R (24 bp) 5′-GACTTCGTTTCCGGTCTTCTCAGG-3′.
SKN-1F (23 bp) 5′-CATCGTCATACGATCGGATCACG-3′,
SKN-1R (22 bp) 5′-GTAGGCGTAGTTGGATGTTGGG-3′.
PAL-1F (23 bp) 5′-CTGAGAGAAAAGATGCTGCAACC-3′,
PAL-1R (22 bp) 5′-AAATGGATCCGTTCAGAGTGGG-3′.

Results

HLH-1 is sufficient to activate the body wall muscle-like program

To test the myogenic potential of HLH-1 in the context of *C. elegans* development, we engineered the full-length *hlh-1* cDNA coding region under the control of a heat shock promoter (Stringham et al., 1992) and created integrated transgenic nematode strains harboring these constructs. After incubation at room temperature for various periods of time, isolated transgenic embryos were heat shocked (34°C) for 30 minutes and assayed over time for the expression of cell type-specific markers.

Under optimal conditions, heat shock-induced *hlh-1* was robust and high levels of nuclear localized protein could be detected in most cells of the embryos within 30 minutes after the end of heat shock induction. Levels of HLH-1 remained high throughout embryogenesis, although nuclear localization was less pronounced after overnight (16-20 hours) incubation of the treated embryos. Embryos incubated overnight arrested with 400-500 cells and appeared healthy, as assayed by Nomarski optics, indicating that neither the heat shock treatment nor high HLH-1 levels had any obvious deleterious effect on cellular viability or proliferation.

In almost all treatment paradigms, over-expression of *hlh-1* resulted in widespread myogenesis; most cells had adopted a muscle-like fate. The muscle marker routinely used to assay myogenesis was myosin heavy chain A [MHC A (Miller et al., 1986) Fig. 1]. Although high levels of MHC A were detected, filaments were disorganized and these muscle-like cells fail to contract. To determine the extent to which these cells adopted a true muscle-like fate, we tested several additional muscle markers, including several structural proteins. The muscle-like cells resulting from heat shock-induced HLH-1 activated a *myo-3::gfp* reporter gene (Fire and Waterston, 1989) and were positive for filamentous actin, UNC-89 (Benian et al., 1996), UNC-98 (Mercer et al., 2003) and PAT-3 (Gettner et al., 1995; Francis and Waterston, 1985) (data not shown). These muscle cells were not positive for the pharyngeal muscle-specific marker 3NB12 (Priess and Thomson, 1987). The continued presence of HLH-1 and activation of several body wall muscle markers is consistent with cells terminally differentiating and adopting a fate most closely resembling body wall muscle cells.

HLH-1 apparently acts in a feed-back loop to maintain *hlh-1* gene expression (Krause et al., 1994). To determine if ectopic HLH-1 was functioning alone to drive myogenesis or acting through the endogenous *hlh-1* gene, we crossed the heat-shock-driven *hlh-1* strain into a balanced *hlh-1(cc450)* null mutant background (Chen et al., 1992). Activation of HLH-1 in animals lacking the endogenous *hlh-1* gene resulted in widespread myogenesis in all embryos, indistinguishable from non-mutant controls (data not shown). This demonstrated that a single pulse of heat-shock-activated HLH-1 was able to drive myogenesis in the absence of endogenous *hlh-1* gene activity.

The excess number of muscle-like cells observed after ectopic activation of HLH-1 could be due to excessive proliferation of myogenic blastomeres, conversion of other cell types to muscle, or both. The total number of cells in HLH-1-activated, terminally arrested embryos was similar to that normally born during embryogenesis, suggesting that there was not a general and widespread hyper-proliferation of blastomeres induced by HLH-1. We therefore assayed the number of embryonic cells adopting one of several cell fates

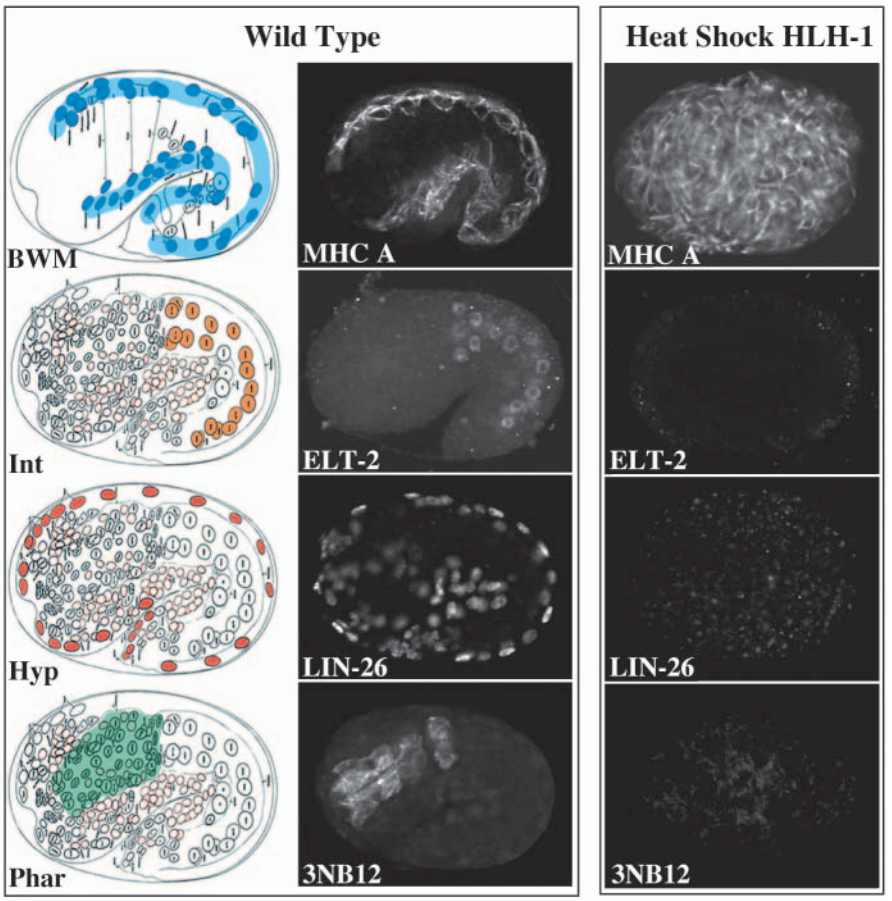

Fig. 1. Ectopic HLH-1 activity converts most somatic blastomeres to a body wall muscle-like fate. (Left) Diagrams of wild-type embryos at the comma stage of development [adapted from Sulston et al. (Sulston et al., 1983)] with four different cell type patterns highlighted by nuclei or tissue area shading; body wall muscle (BWM), intestine (Int); hypodermis (Hyp) and pharynx (Phar). Adjacent to each diagram is a wild-type embryo stained with an antibody recognizing the corresponding tissue and imaged in a single focal plane. The far right column shows terminally arrested embryos in which HLH-1 activity was induced in early development by heat shock treatment. All embryos showed widespread myogenesis as indicated by MHC A staining in a representative focal plane from throughout the embryo (top) and a complete lack of staining for markers of the intestine, hypodermis and pharynx (below). All images in this and subsequent figures are a single focal plane deconvoluted using Hyguens software.

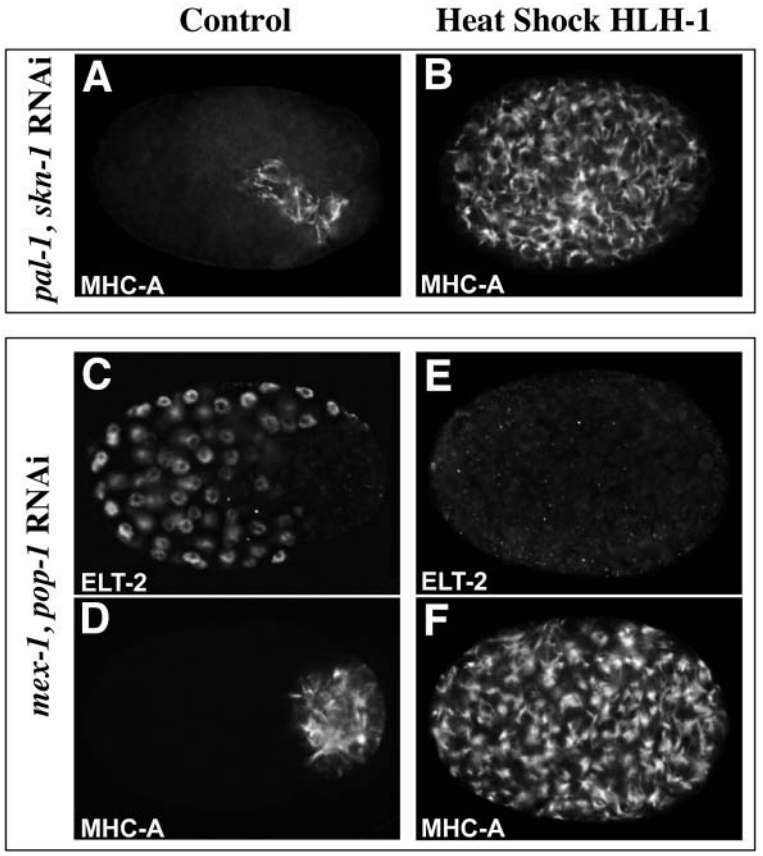

Fig. 2. The myogenic activity of HLH-1 in embryos lacking normal cell fate specification. All embryos shown are transgenic for the heat shock promoter-driven *hlh-1* transgene that is inactive in controls (left) or activated by a heat pulse (right). Top: embryos were depleted of PAL-1 and SKN-1 activity by RNAi, allowed to develop overnight, and stained for MHC A. Control embryos (A) have only a small amount of muscle due to zygotic PAL-1 activity that is not completely eliminated by RNAi (Edgar et al., 2001). In the presence of ectopic HLH-1 activity (B), most cells in *pal-1, skn-1* double RNAi-treated embryos strongly express MHC A as shown by this focal plane representative of the entire embryo. Bottom: embryos were treated with *mex-1, pop-1* double RNAi to convert a majority of anterior blastomeres to ELT-2-positive intestinal cells (C); a small area of MHC A-positive muscle remains in these embryos (D). Heat shock-induced HLH-1 activity, in combination with *mex-1, pop-1* double RNAi treatment, results in a complete loss of ELT-2 staining (E) and strong MHC A staining (F) in most blastomeres.

by scoring them for LIN-26 (hypodermis) (Labouesse et al., 1996), ELT-2 (intestine) (Fukushige et al., 1998), 3NB12 (pharyngeal muscle) (Priess and Thomson, 1987) and P-granules (germline) (Strome and Wood, 1983) following heat shock induction of HLH-1. Although the majority of activated HLH-1 embryos had the normal number of germline precursors (two), there was a complete elimination of all somatic cell types assayed in almost all embryos (Fig. 1). Similar heat shock of wild-type embryos did not affect normal development and these embryos were positive for all cell-type markers tested. These results suggested that early expression of *hlh-1* was able to convert most, if not all, somatic cells of the embryo into muscle-like cells.

It was possible that HLH-1 was not actively converting cells to muscle but instead blocking normal development and revealing a default muscle cell fate program of early blastomeres. To address this, we eliminated two maternal factors needed to specify several founder blastomere fates. In wild-type embryos, the loss of *skn-1* and *pal-1* gene products prevents the proper specification of the MS, C and D lineages and greatly reduces, or eliminates, the cell types derived from each lineage (Fig. 2) (Bowerman et al., 1992; Bowerman et al., 1993; Hunter and Kenyon, 1996). Such embryos arrest with 400-500 cells, most of which fail to express body wall muscle markers. In HLH-1-activated embryos that have also been depleted of both *skn-1* and *pal-1* gene products by RNAi, almost all cells adopted the body wall muscle-like fate (Fig. 2). These results further demonstrated the myogenic potential of HLH-1 activity and that myogenesis is a consequence of HLH-1 activity.

As an additional assay of cell fate conversion, we tested the function of ectopic HLH-1 in embryos in which most cells had been specified as intestine. Loss of the maternal factor MEX-1 results in the four granddaughters of AB adopting an EMS-like fate (Mello et al., 1992; Schnabel et al., 1996). If these embryos are also depleted of POP-1/TCF, all daughters of the EMS and pseudo-EMS cells will develop like E, transforming the entire anterior of the embryo into intestine (Lin et al., 1995; Maduro et al., 2001). When we ectopically activated HLH-1 in embryos depleted of *mex-1* and *pop-1* by RNAi, almost all somatic cells adopted a muscle-like fate (Fig. 2). Even in *mex-1, pop-1* double RNAi-treated embryos that had been incubated for 150 minutes (~3 hours post-fertilization) prior to HLH-1 activation by heat shock, 80% (n=31) of the embryos had widespread myogenesis and no intestinal cells detectable by ELT-2 antibody staining. This result demonstrates that HLH-1 was sufficient to convert cells destined to become intestine into a muscle-like fate.

Myogenic conversion by HLH-1 acts in a broad window of early development

Stable *hlh-1* gene expression is normally detectable in all body wall muscle precursors shortly after they are born, beginning with the daughters of the D founder about 2 hours after fertilization (Krause et al., 1990). HLH-1 is present in body wall myoblasts as they proliferate and in all differentiated body wall muscle cells in embryos, larvae and adults. To determine if the ability of HLH-1 to convert cells to a muscle-like fate was restricted to a specific time of embryogenesis, embryos harboring the heat shock *hlh-1* transgene were isolated and incubated for various periods of time prior to induction. The percentage of embryos that were positive for MHC A, and each of several different non-body wall muscle cell type markers, was used to determine the efficiency of HLH-1-induced myogenesis. For embryos incubated for more than 90 minutes prior to HLH-1 induction (~2 hours post-fertilization), we used the intestine-specific marker *elt-2*::GFP to count the number of E cell descendants present at the onset of the heat shock under our experimental conditions (Table 1). Embryos that had anywhere between one and eight E cell descendants at the time ectopic HLH-1 activation was initiated showed widespread myogenic

Table 1. Developmental window during embryogenesis in which ectopic HLH-1 activity results in myogenic conversion of blastomeres

	Incubation time prior to heat shock induction of HLH-1			
	45 min	90 min	150 min	210 min
Total cell number	12-24	~50	~112	~300
Int (E) cell number	1-2	2-4	7.9±1.5	10.2±.9
% int-positive embryos (ELT-2 staining)	1.2 (*n*=85)	3.2 (*n*=95)	1.1 (*n*=89)	46.9 (*n*=96)
% hyp-positive embryos (LIN-26 staining)	3.7 (*n*=53)	2.1 (*n*=96)	13.4 (*n*=97)	92.2 (*n*=102)
% phar-positive embryos (3NB12 staining)	6.8 (*n*=73)	4.7 (*n*=106)	6.6 (*n*=90)	25.6 (*n*=90)

conversion and a nearly complete loss of intestine, hypodermis and pharyngeal cell markers (Table 1). At 210 minutes of incubation (~4 hours post-fertilization), when embryos averaged 10.2 E descendants, markers indicative of other cell fates became evident in most embryos. This defined a window of competence for blastomeres to respond to ectopic HLH-1 to the first 3 hours of development, up to the eight E cell stage of embryogenesis. After this time, the ability of non-myogenic cells to respond to HLH-1 declines rapidly over the subsequent hour of development and is lost completely in terminally differentiated cells.

PAL-1 activates *hlh-1* in body wall muscle precursors

PAL-1 is a Caudal-related homeobox protein that is required for the development of the posterior founder blastomeres. Maternal PAL-1 functions to specify both the C and D lineages (Hunter and Kenyon, 1996) while zygotically expressed *pal-1* is needed for proper development of their descendants (Edgar et al., 2001). The C lineage gives rise to hypodermis and body wall muscle, whereas the D lineage gives rise exclusively to body wall muscle cells (Sulston et al., 1983). MEX-3 is a negative regulator of PAL-1 and *mex-3* mutations or administration of *mex-3* RNAi results in anterior blastomeres (the granddaughters of AB) adopting a C-like fate (Draper et al., 1996; Hunter and Kenyon, 1996; Bowerman et al., 1997; Huang et al., 2002). Although there are no other known MEX-3 targets, lineages not expressing PAL-1 are also affected in MEX-3 mutants suggesting additional functions beyond PAL-1 regulation (Draper et al., 1996).

To determine if *hlh-1* is a downstream target of PAL-1 in myogenic lineages, we ectopically activated PAL-1 in early embryos harboring an *hlh-1::gfp* reporter gene. In *mex-3* RNAi-treated embryos, ectopic PAL-1 activated the *hlh-1* reporter within 3 hours in a subset of blastomeres that differentiated as body wall muscle-like cells. The extent of differentiation of muscle-like cells in mex-3 RNAi-treated embryos was indistinguishable from that observed after heat shock induction of HLH-1 activity. The embryos also had ectopic hypodermal cells that were LIN-26 positive, as was expected from previous characterizations of *mex-3* mutants (Draper et al., 1996; Bowerman et al., 1997). To determine if the effects of *mex-3* RNAi were attributable to PAL-1 mis-regulation, we also ectopically over-expressed PAL-1 using a heat shock promoter-driven *pal-1* cDNA clone, kindly provided by Julie Ahringer. We found that heat shock-induced PAL-1 activity in early embryos also resulted in widespread *hlh-1* reporter gene activation and myogenesis (Fig. 3), as well as hypodermal development (data not shown). Although similar, the effects of *mex-3* RNAi and heat shock-induced PAL-1 were not identical and could be distinguished with the gut cell marker ELT-2. All *mex-3* RNAi-treated embryos (100%, *n*=16) were positive for ELT-2 demonstrating that the gut lineage was largely unaffected in these embryos, consistent

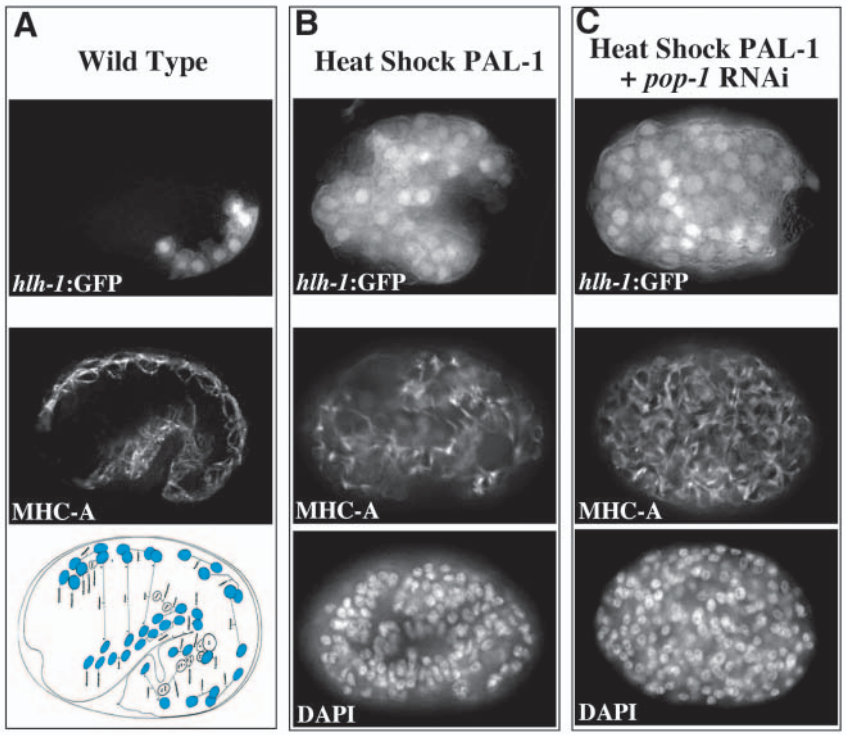

Fig. 3. Ectopic PAL-1 activates HLH-1 and the body wall muscle-like developmental program. The left panels (A) show a wild-type embryo with the body wall muscle cells at 3 hours post-fertilization highlighted by *hlh-1::gfp* expression (top); descendants of the C and D lineage are visible at this time. A comma stage embryo stained for MHC A (middle) shows the left side focal plane of body wall muscle quadrants; a corresponding diagram is shown below [adapted from Sulston et al. (Sulston et al., 1983)]. The center column of panels (B) show the response of muscle markers to heat shock-induced PAL-1 activity. Strong activation of *hlh-1::gfp* occurs within 3 hours of treatment in many cells (top). After overnight incubation, most cells in the embryo are MHC A positive (middle) when compared with the DAPI image of the same embryo (bottom). The right set of panels (C) show that the depletion of POP-1 activity by RNAi enhances the myogenic activity of ectopic PAL-1 (compare to B). Embryo staging and staining in C are the same as those in B, and images in these panels are representative of most focal planes of the embryo.

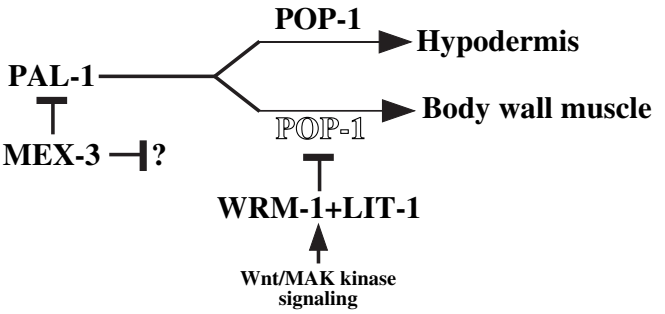

Fig. 4. A schematic representation of the previously published (see text) interactions of several factors in the early embryo. PAL-1, which is negatively regulated by MEX-3, can specify a hypodermal or body wall muscle fate, depending on the level of POP-1. In the presence of high POP-1 (solid black letters), PAL-1 activity results in a hypodermal-like fate. POP-1 can be down-regulated (open lettering) via Wnt/MAP kinase signaling through the combined action of the factors WRM-1 and LIT-1 (see text). PAL-1 specifies a body wall muscle fate when POP-1 levels are low or absent.

with earlier studies (Draper et al., 1996). However, only 58% (n=268) of heat shock-induced PAL-1 embryos were ELT-2 positive, suggesting that over-expression of PAL-1 interfered with normal gut development.

PAL-1-induced, but not HLH-1-induced, myogenesis is blocked by POP-1

The cells of the C lineage that give rise to hypodermis and body wall muscle are the result of anterior-posterior cell divisions (Sulston et al., 1983) and have different levels of nuclear POP-1/TCF (Lin et al., 1998). Studies of Wnt/MAP kinase signaling in the early embryo have shown that LIT-1/MAP kinase phosphorylation of POP-1/TCF, in a WRM-1/beta-catenin-dependent manner, results in the nuclear export of POP-1 (Kaletta et al., 1997; Lin et al., 1998; Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999; Lo et al., 2004). Both LIT-1 (Kaletta et al., 1997) and POP-1 (Mickey, 2000) are involved in cell fate regulation in the C descendants; hypodermal precursors have high POP-1 levels whereas myogenic precursors have low POP-1 levels reflecting the actions of Wnt/MAP kinase signaling (Fig. 4).

To test the effects of Wnt/MAPK signaling in our experimental system, we repeated our ectopic PAL-1 activity experiments in embryos in which POP-1 levels were knocked down in all blastomeres by RNAi. Ectopic PAL-1 activity in these experiments was achieved using *mex-3* RNAi. As predicted, reduction (or loss) of POP-1 greatly enhanced the ability of ectopic PAL-1 to activate HLH-1 and the muscle-like fate (Fig. 3). Muscle-like conversion in these treated embryos was comparable to embryos in which ectopic HLH-1 alone had been activated; almost all blastomeres adopted a muscle-like fate. Conversely, when we blocked the down-regulation of POP-1/TCF using RNAi knockdown of LIT-1/MAP kinase (or WRM-1/β-catenin; data not shown), we found that ectopic PAL-1 activated a hypodermal-like program in almost all somatic blastomeres (Fig. 5). To determine if the effects of Wnt/MAP kinase signaling were POP-1-dependent, we heat shock-induced ectopic PAL-1 in embryos depleted of both LIT-1 and POP-1 activity by RNAi. We assayed myogenesis using an integrated *hlh-1::gfp* transgene. As seen with *pop-1* RNAi alone, *lit-1* and *pop-1* double RNAi, in combination with ectopic PAL-1, resulted in *hlh-1::gfp* reporter gene activation in most somatic cells for 84% (n=44) of the embryos. These results demonstrated that PAL-1 is myogenic and activates *hlh-1* in somatic cells, provided that they lack POP-1 or that POP-1 has been down-regulated by Wnt/MAP kinase signaling. PAL-1-positive blastomeres with high POP-1 activity adopt a hypodermal-like fate (see Fig. 4).

Wnt/MAP kinase signaling has also been implicated in the formation of body wall muscle cells from the MS lineage from previous studies of *lit-1* (Kaletta et al., 1997). To explore this possibility further, we increased the number of MS-like blastomeres using *mex-1* RNAi, which causes a transformation of AB granddaughter blastomeres to an MS-like lineage, in addition to other lineage defects (Mello et al., 1992; Schnabel et al., 1996). As a consequence of the reiteration of the MS lineage there is a large excess (four- to fivefold) of body wall and pharyngeal muscle that can be distinguished from each other using antibodies to MHC A and 3NB12, respectively. Consistent with previous studies (Kaletta et al., 1997), blocking MAP kinase signaling using *lit-1* RNAi in a *mex-1* RNAi background resulted in a severe

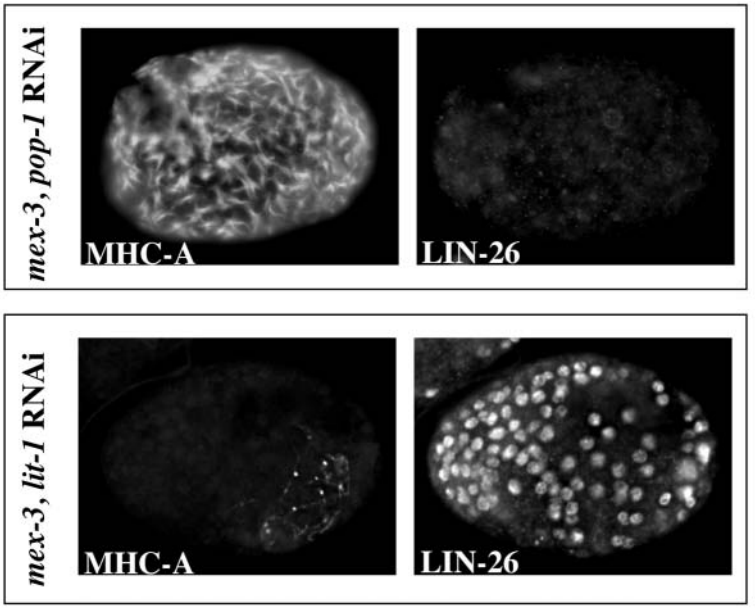

Fig. 5. High POP-1 blocks the myogenic activity, and promotes the hypodermal activity, of PAL-1. All embryos were treated with *mex-3* RNAi that results in ectopic PAL-1 activity. After overnight incubation, embryos were assayed by antibody staining for the muscle marker MHC A and the hypodermal marker LIN-26. The upper pair of panels show representative focal planes of the robust myogenesis (MHC A) accompanying ectopic PAL-1 activity after co-depletion of POP-1 activity by RNAi; no LIN-26-positive cells are detected. In contrast, the lower pair of panels shows an embryo that was co-depleted of LIT-1 by RNAi to block the down-regulation of POP-1 by Wnt/MAP kinase signaling (see text). After overnight incubation, only a few cells (presumably from the D lineage) adopted a muscle-like fate whereas many cells adopted a hypodermal-like fate as seen in these images that are representative of focal planes throughout the embryo.

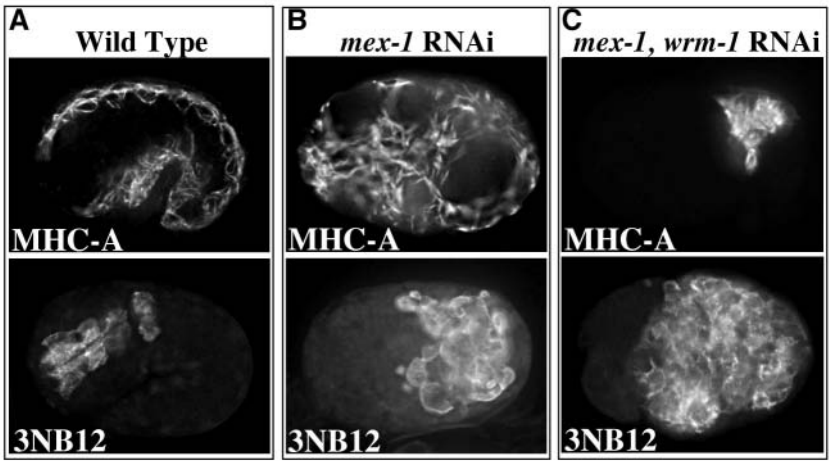

Fig. 6. Wnt/MAP kinase signaling plays a role in MS-derived body wall muscle development. The left column (A) shows the comma stage pattern of body wall muscle (MHC A) and pharyngeal muscle (3NB12) in a wild-type embryo. Embryos depleted of MEX-1 activity (B) have a large excess in body wall and pharyngeal muscle due to cell fate transformations that causes a reiteration of MS-like lineages (Mello et al., 1992). The right column (C) shows the effects of blocking Wnt/MAP kinase signaling using *wrm-1* RNAi, in an embryo co-depleted of *mex-1*. There is a severe reduction in the number of body wall muscle-like cells and concomitant increase in pharyngeal-like muscle cells demonstrating the role of Wnt/MAP kinase signaling in body wall formation within the MS lineage. Images in B and C are representative of focal planes throughout the embryo.

loss of body wall muscle and a large increase in pharyngeal muscle. To confirm that this effect was also WRM-1/beta-catenin dependent, we assayed *mex-1*, *wrm-1* double RNAi embryos for muscle production and obtained similar results to those seen with *mex-1, lit-1* double RNAi (Fig. 6). Most embryos had a small cluster of body wall muscle cells (~10) near the posterior of the embryo; these are probably descendants of D, which should be largely unaffected by *lit-1* or *wrm-1* RNAi as D has no detectable POP-1 (Lin et al., 1998) and only low levels of POP-1 are detected in D descendants (data not shown). *mex-1* RNAi perturbs both the C and D lineages, in addition to affecting AB (Schnabel et al., 1996), explaining why we did not observed the normal number (20) of D descendants. Our results confirm that Wnt/MAPK signaling is required to knock down POP-1 levels in cells destined to be body wall muscle within the MS lineage.

To determine if HLH-1 function was also dependent on Wnt/MAP kinase signaling, we compared ectopic myogenesis after heat shock induction of HLH-1 in a wild-type versus *lit-1* RNAi background. Loss of *lit-1* gene activity had no effect on the ability of HLH-1 to induce widespread myogenesis, demonstrating that HLH-1 can function independently of Wnt/MAP kinase signaling (data not shown).

The ability of PAL-1 to induce muscle is independent of HLH-1 activity

In all experiments in which ectopic PAL-1 activity, in concert with low or no POP-1 activity, resulted in a muscle-like fate, HLH-1 was activated and localized to the nucleus of myogenic cells prior to the expression of terminal muscle markers. Previous genetic studies have demonstrated that HLH-1 is not necessary for cells to adopt a body wall muscle fate during embryogenesis (Chen et al., 1992; Chen et al., 1994). To determine if the PAL-1-induced muscle-like fate was dependent on HLH-1, we repeated the ectopic PAL-1 experiments in an *hlh-1* null mutant background. Hermaphrodites heterozygous for the balanced *hlh-1(cc450)* null allele (Chen et al., 1992) were treated with *mex-3*, *pop-1* double RNAi and progeny embryos collected. If HLH-1 was required for PAL-1 to induce muscle, 25% of the embryos (corresponding to the homozygous *hlh-1(cc450)* animals) should not respond to PAL-1. However, all treated embryos resulted in widespread body wall muscle-like myogenesis demonstrating that HLH-1 activity was not required for PAL-1-induced myogenesis (Fig. 7). We did notice that 22% (n=196) of the embryos showed less robust myogenesis, based on MHC A filament formation (Fig. 7B). These experiments

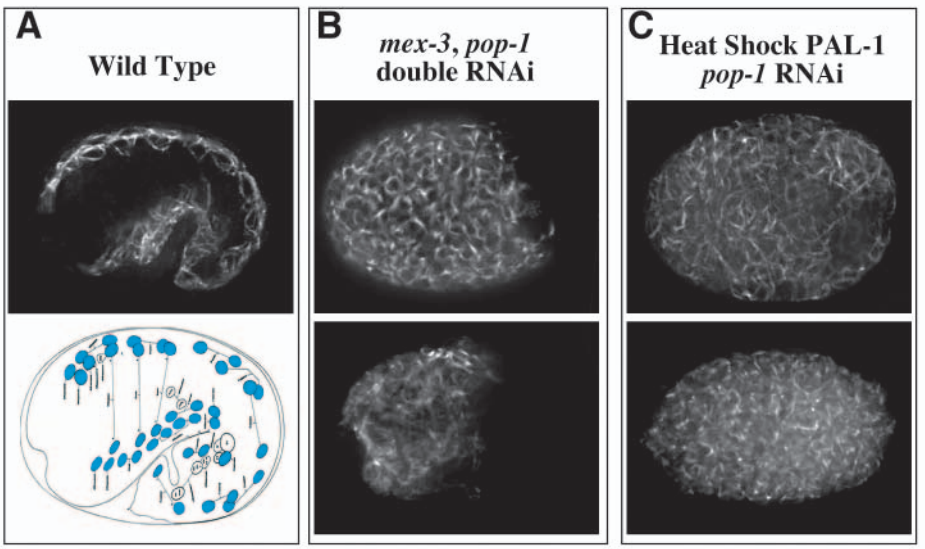

Fig. 7. Ectopic PAL-1 activates the muscle-like program independent of HLH-1. The left panels (A) shows the MHC A pattern on the left side of a comma stage wild-type embryo (top) and a diagram of the body wall muscle nuclei (bottom; adapted from Sulston et al., 1983). In the center (B), treatment of wild-type embryos (top) with *mex-3, pop-1* double RNAi results in widespread myogenesis as indicated by MHC A staining. The same treatment in a presumed *hlh-1(cc450)* mutant null genetic background (bottom: see text for details) results in similar amounts of ectopic muscle, although MHC A expression appears weaker. The right panels (C) show similar results in embryos treated with heat shock-induced PAL-1 and *pop-1* RNAi in either a wild-type (top) or an *hlh-1* RNAi (bottom) embryo. Images in B and C are representative of focal planes throughout the embryo.

were repeated using the temperature-sensitive *hlh-1* allele *cc561* (Harfe et al., 1998a), or *hlh-1* RNAi for which the genotype of each embryo was unambiguous and the same results were obtained (Fig. 7C); myogenesis occurred but was not as robust in the absence of HLH-1. These results demonstrated that ectopic PAL-1-induced myogenesis was slightly more robust with, but not dependent on, HLH-1.

Discussion

HLH-1 is a myogenic regulator

The hallmark feature of myogenic regulatory factors (MRFs) is their ability to convert cultured non-muscle cell types into striated muscle-like cells. This study provides the first evidence in *C. elegans* that HLH-1 is myogenic and clearly demonstrates that this factor is a bona fide MRF. Induction of HLH-1 activity throughout the early *C. elegans* embryo is sufficient to convert most, if not all, somatic cells into a body wall muscle-like fate as assayed by the expression of several cell-type-specific markers.

The myogenic activity of HLH-1 is robust, possibly reflecting its ability to auto-activate its expression in a positive feedback loop (Krause et al., 1994). We find that induction of HLH-1 as late as the eight E cell (>100 total cells) stage of embryogenesis still results in almost all somatic cells adopting a muscle-like fate. Within the E lineage, several tissue-specific markers (e.g. *end-1* and *elt-2*) are being expressed by the eight E cell stage, indicating that these cells have already initiated the intestinal cell fate (reviewed by Maduro and Rothman, 2002). The over-expression of HLH-1 is able to extinguish the gut program and redirect these early intestinal cells into body wall muscle-like cells. This distinguishes our studies from previous work in which blastomere fate-switching was induced much earlier in development (two E cell stage) (Fukushige et al., 1998; Zhu et al., 1998). We did not observe cells expressing terminal markers of multiple fates, suggesting that cell fate decisions are mutually exclusive. How competing transcriptional factors result in all-or-none developmental fate decisions at the mechanistic level is an interesting and unanswered question. Regardless of the mechanism, the potency of HLH-1 reveals a remarkable level of developmental plasticity in cells that have already initiated a cell fate program; within the first 3 hours of development somatic blastomeres are not irreversibly committed to a single fate.

The decision to adopt a body wall muscle cell fate can be cell autonomous. The blastomeres adopting the muscle-like fate are not related by lineage and are not in a fixed location within the embryo. If exogenous signals are required for HLH-1-mediated myogenesis, they must originate in the germline precursors as that is the only non-muscle lineage that remains identifiable in these HLH-1-activated embryos. Such signals would have to be far-reaching to affect the most anterior blastomeres that do not physically contact the P cell lineage after the four cell stage. Consequently, we think it is unlikely that signals from outside the muscle lineage are required for HLH-1 to activate the muscle program, although we can not exclude a ‘community effect’ (Gurdon, 1988) among muscle cells.

One question that arises from our current work is the extent to which HLH-1 is able to drive myogenesis. That is, are these muscle-like cells exhibiting most of the characteristics of terminally differentiated cells, or have they merely initiated a small part of the muscle program? Studies in both *Xenopus* (Hopwood and Gurdon, 1990; Hopwood et al., 1991) and the mouse (Miner et al., 1992; Faerman et al., 1993) have demonstrated that ectopic MRF activity in vivo is able to activate some genes of the skeletal muscle program but fails to drive terminal muscle differentiation. However, more recent work has shown terminal differentiation of skeletal muscle after transfection of the chicken embryonic neural tube with Myf5 or MyoD expression transgenes (Delfini and Duprez, 2004). We have assayed five major markers of body wall muscle in *C. elegans* and found that all are present in the muscle-like cells generated by ectopic HLH-1. This includes gene products encoding structural components needed for terminal differentiation. However, these muscle-like cells lack clearly defined sarcomeres and contraction has not been observed. This may reflect the fact that these embryos also lack somatic non-muscle cell types that might be important for normal sarcomere assembly and function. This includes the hypodermal cells, which play a role in sarcomere organization (reviewed by Rogalski et al., 2001; Labouesse and Georges-Labouesse, 2003) and neurons needed for innervation. Additional studies will be required to determine if the failure to make functional sarcomeres reflects the lack of an appropriate cellular environment, a failure in expression in all requisite muscle cell genes, or a combination of these factors.

PAL-1 is sufficient for myogenesis in cells with little or no POP-1 activity

Our results demonstrate that PAL-1 is sufficient to activate *hlh-1* and that this is part of the mechanism of body wall muscle development in the C and D lineages. Interestingly, this function of PAL-1 is not completely HLH-1 dependent, demonstrating that one or more factors must act redundantly with HLH-1 in driving body wall myogenesis. It is not clear if PAL-1 directly activates *hlh-1*. The *hlh-1* promoter has been extensively characterized and essential cis-acting elements for expression delineated (Krause et al., 1994) (J. Liu, personal communication). In addition, the DNA-binding site preferences for Caudal and related factors have been defined in *Drosophila* and mammalian tissue culture studies (Dearolf et al., 1989; Suh et al., 1994; Charite et al., 1998; Xu et al., 1999). However, we have yet to uncover a direct interaction between PAL-1 and the *hlh-1* promoter using either bioinformatic or experimental approaches. This analysis is complicated by the AT-rich binding site preferences of Caudal-related factors and our lack of understanding of which, if any, co-factors act in concert with PAL-1 to regulate transcription in *C. elegans*. This is an important question that needs to be answered in future studies.

The effects of PAL-1 on cell fate specification are altered in the presence of POP-1 activity. Cells lacking POP-1 (e.g. the D lineage) respond to PAL-1 by activating the body wall muscle program. However, in the presence of POP-1 activity, PAL-1 instead promotes hypodermal fate. Our results show that POP-1 activity must be down-regulated in cells that become body wall muscle in both the C and MS lineages. The down-regulation of POP-1 via Wnt/MAP kinase signaling is, therefore, an important component of embryonic body wall muscle development in all lineages except D. The source(s) of

Wnt/MAP kinase signaling to descendants of the C lineage is unknown. However, once *hlh-1* is activated within body wall muscle precursors, Wnt/MAP kinase signaling is dispensable.

Mechanistically, the combination of PAL-1 and Wnt/MAP kinase signaling in the C lineage acts in a manner that is analogous to E and MS founder fate specification. The transcription factor SKN-1 is required for both the MS and E founder fates that give rise to mesoectoderm and endoderm, respectively (Bowerman et al., 1992; Bowerman, 1995). In the absence of POP-1, SKN-1 initiates a gut cell fate, analogous to PAL-1 initiating a muscle-like fate. In the presence of high POP-1 activity, SKN-1 results in the MS fate, analogous to PAL-1 resulting in a hypodermal fate. In both cases, Wnt/MAP kinase signaling is responsible for the down-regulation of POP-1 (Rocheleau et al., 1997; Thorpe et al., 1997; Lin et al., 1998; Meneghini et al., 1999).

The anatomically simple body wall musculature arises from a complex genetic program

The body wall muscle cells are the only striated musculature in *C. elegans*. All 81 embryonically born body wall muscle cells are arranged along the length of the animal in one of four parallel quadrants (Waterston, 1988). These cells are morphologically nearly identical to each other, making *C. elegans* body wall muscle one of the simplest striated muscle systems under study. Despite this anatomical simplicity, these 81 cells arise by a surprisingly complex number of different genetic programs. There are at least two maternal transcription factors, SKN-1 and PAL-1, regulating embryonic myogenesis in a manner that is distinct from each other and distinct from the regulation of post-embryonic body wall muscle development (Bowerman et al., 1992; Hunter and Kenyon, 1996; Edgar et al., 2001). In addition, ablation experiments reveal a complicated interplay between different founder blastomere lineages in regulating myogenesis (Schnabel, 1995). Finally, blastomere culture experiments reveal that cell-cell interactions within the C lineage influence cell fate decisions (Mickey, 2000). Taken together, these studies reveal a surprising level of complexity for the genesis of an anatomically simple striated musculature.

Similarities between myogenesis in *C. elegans* and vertebrates

Previous studies of the transcriptional regulation of body wall myogenesis in *C. elegans* have highlighted numerous differences between the vertebrate and nematode systems. In vertebrates, MRFs heterodimerize with members of the broadly distributed E protein family to activate transcription of muscle-specific genes (reviewed by Weintraub et al., 1991; Weintraub, 1993). In *C. elegans*, the only E-related factor, E/DA, is not detected in striated muscle cells and HLH-1 appears to function as a homodimer to activate transcription (Krause et al., 1997). In addition, members of the Twist and MEF-2 transcription factor families, which play important roles in vertebrate mesoderm specification and muscle differentiation (reviewed by Black and Olson, 1998; Castanon and Baylies, 2002), play little or no apparent role in embryonic body wall muscle development in *C. elegans* (Corsi et al., 2000; Dichoso et al., 2000). The transcriptional cascade regulating myogenesis in *C. elegans* embryogenesis is clearly distinct from that operating in the vertebrates.

However, the current study also highlights the similarities between nematode and vertebrate striated myogenesis. HLH-1 is a bona fide MRF with potent myogenic activity in vivo, demonstrating conservation of function throughout evolution. It would appear that function is redundant to other, as yet unidentified, factors in *C. elegans* and *Drosophila* whereas vertebrates may rely predominantly on the redundancy provided by multiple MRFs.

The role of Wnt signaling in *C. elegans* body wall muscle specification also suggests parallels to other systems. During vertebrate embryogenesis, Wnts are needed for the dorsal neural tube and surface ectoderm to induce myogenesis in the adjacent somites in the trunk region (Munsterberg et al., 1995; Tajbakhsh et al., 1998). Wnt signaling is also important for activating myogenesis in resident stem cells during muscle regeneration following injury in mice (Polesskaya et al., 2003). During both of these vertebrate developmental events, Wnt signaling precedes the activation of the MRFs. *C. elegans* Wnt/MAPK signaling similarly precedes expression of *hlh-1*, suggesting that at least some aspects of the regulation of MRF genes may also be evolutionarily conserved.

We thank Julie Ahringer, Guy Benian, Andy Fire, Michel Labouesse, Rueyling Lin, Jim McGhee and Don Moerman for useful strains and reagents. Thanks to Andy Golden, Joan McDermott, Jim McGhee, Geraldine Seydoux and anonymous reviewers for comments on improving the manuscript.

References

Ahringer, J. (1997). Maternal control of a zygotic patterning gene in *Caenorhabditis elegans*. **124**, 3865-3869.

Balagopalan, L., Keller, C. A. and Abmayr, S. M. (2001). Loss-of-function mutations reveal that the *Drosophila nautilus* gene is not essential for embryonic myogenesis or viability. *Dev. Biol.* **231**, 374-382.

Benian, G. M., Tinley, T. L., Tang, X. and Borodovsky, M. (1996). The *Caenorhabditis elegans* gene *unc-89*, required for muscle M-line assembly, encodes a giant modular protein composed of Ig and signal transduction domains. *J. Cell Biol.* **132**, 835-848.

Berkes, C. A., Bergstrom, D. A., Penn, B. H., Seaver, K. J., Knoepfler, P. S. and Tapscott, S. J. (2004). Pbx marks genes for activation by MyoD indicating a role for a homeodomain protein in establishing myogenic potential. *Mol. Cell* **14**, 465-477.

Black, B. L. and Olson, E. N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF-2) proteins. *Ann. Rev. Cell Dev. Biol.* **14**, 167-196.

Bowerman, B. (1995). Determinants of blastomere identity in the early *C. elegans* embryo. *BioEssays* **17**, 405-414.

Bowerman, B., Eaton, B. A. and Priess, J. R. (1992). *skn-1*, a maternally expressed gene required to specify the fate of ventral blastomeres in the early *C. elegans* embryo. *Cell* **68**, 1061-1075.

Bowerman, B., Draper, B. W., Mello, C. C. and Priess, J. R. (1993). The maternal gene *skn-1* encodes a protein that is distributed unequally in early *C. elegans* embryos. *Cell* **74**, 443-452.

Bowerman, B., Ingram, M. K. and Hunter, C. P. (1997). The maternal *par* genes and the segregation of cell fate specification activities in early *Caenorhabditis elegans* embryos. *Development* **124**, 3815-3826.

Buckingham, M. (2001). Skeletal muscle formation in vertebrates. *Curr. Opin. Genet. Dev.* **11**, 440-448.

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D. and Relaix, F. (2003). The formation of skeletal muscle: from somite to limb. *J. Anat.* **202**, 59-68.

Castanon, I. and Baylies, M. K. (2002). A Twist in fate: evolutionary comparison of Twist structure and function. *Gene* **287**, 11-22.

Charite, J., deGraaff, W., Consten, D., Reijnen, M. J., Korving, J. and Deschamps, J. (1998). Transducing positional information to the *Hox* genes: critical interaction of *cdx* gene products with position-sensitive regulatory elements. *Development* **125**, 4349-4358.

Chen, L., Krause, M., Draper, B., Weintraub, H. and Fire, A. (1992). Body-wall muscle formation in *Caenorhabditis elegans* embryos that lack the MyoD homolog *hlh-1*. *Science* **256**, 240-243.

Chen, L., Krause, M., Sepanski, M. and Fire, A. (1994). The *Caenorhabditis elegans* MyoD homologue HLH-1 is essential for proper muscle function and complete morphogenesis. *Development* **120**, 1631-1641.

Corsi, A. K., Kostas, S. A., Fire, A. and Krause, M. (2000). *Caenorhabditis elegans* Twist plays an essential role in non-striated muscle development. *Development* **127**, 2041-2051.

Dearolf, C. R., Topol, J. and Parker, C. S. (1989). The *caudal* gene product is a direct activator of *fushi tarazu* transcription during Drosophila embryogenesis. *Nature* **341**, 340-343.

Delfini, M.-C. and Duprez, D. (2004). Ectopic Myf5 or MyoD prevents the neuronal differentiation program in addition to inducing skeletal muscle differentiation, in the chick neural tube. *Development* **131**, 713-723.

Dichoso, D., Brodigan, T., Chwoe, K. Y., Lee, J. S., Llacer, R., Park, M., Corsi, A. K., Kostas, S. A., Fire, A., Ahnn, J. and Krause, M. (2000). The MADS-box factor CeMEF2 is not essential for *Caenorhabditis elegans* myogenesis and development. *Dev. Biol*. **223**, 431-440.

Draper, B. W., Mello, C. C., Bowerman, B., Hardin, J. and Priess, J. R. (1996). MEX-3 is a KH domain protein that regulates blastomere identity in early *C. elegans* embryos. *Cell* **87**, 205-216.

Edgar, L. G., Carr, S., Wang, H. and Wood, W. B. (2001). Zygotic expression of the *caudal* homolog *pal-1* is required for posterior patterning in *Caenorhabditis elegans* embryogenesis. *Dev. Biol.* **229**, 71-88.

Egan, C. R., Chung, M. A., Allen, F. L., Heschl, M. F., Van Buskirk, E. L. and McGhee, J. D. (1995). A gut-to-pharynx/tail switch in embryonic expression of the *Caenorhabditis elegans ges-1* gene centers on two GATA sequences. *Dev. Biol.* **170**, 397-419.

Faerman, A., Pearson-White, S., Emerson, C. and Shani, M. (1993). Ectopic expression of MyoD1 in mice causes prenatal lethalities. *Dev. Dyn*. **196**, 165-173.

Fire, A. and Waterston, R. H. (1989). Proper expression of myosin genes in transgenic nematodes. *EMBO J.* **8**, 3419-3428.

Francis, G. R. and Waterston, R. H. (1985). Muscle organization in *Caenorhabditis elegans*: localization of proteins implicated in thin filament attachment and I-band organization. *J. Cell Biol.* **101**, 1532-1549.

Fukushige, T., Hawkins, M. G. and McGhee, J. D. (1998). The GATA-factor ELT-2 is essential for formation of the *Caenorhabditis elegans* intestine. *Dev. Biol.* **198**, 286-302.

Gettner, S. N., Kenyon, C. and Reichardt, L. F. (1995). Characterization of beat pat-3 heterodimers, a family of essential integrin receptors in *C. elegans*. *J. Cell Biol.* **129**, 1127-1141.

Gurdon, J. B. (1988). A community effect in animal development. *Nature* **336**, 772-774.

Harfe, B. D., Branda, C. S., Krause, M., Stern, M. J. and Fire, A. (1998a). MyoD and the specification of muscle and non-muscle fates during postembryonic development of the *C. elegans* mesoderm. *Development* **125**, 2479-2488.

Harfe, B. D., Vaz Gomes, A., Kenyon, C., Liu, J., Krause, M. and Fire, A. (1998b). Analysis of a *Caenorhabditis elegans* Twist homolog identifies conserved and divergent aspects of mesodermal patterning. *Genes Dev.* **12**, 2623-2635.

Hopwood, N. D. and Gurdon, J. B. (1990). Activation of muscle genes without myogenesis by ectopic expression of MyoD in frog embryo cells. *Nature* **347**, 197-200.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1991). *Xenopus* Myf-5 marks early muscle cells and can activate muscle gene ectopically in early embryos. *Development* **111**, 551-560.

Huang, N. N., Mootz, D. E., Walhout, A. J. M., Vidal, M. and Hunter, C. P. (2002). MEX-3 interacting proteins link cell polarity to asymmetric gene expression in *Caenorhabditis elegans*. *Development* **129**, 747-759.

Hunter, C. P. and Kenyon, C. (1996). Spatial and temporal controls target *pal-1* blastomere-specification activity to a single blastomere lineage. *Cell* **87**, 217-226.

Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Cleavers, H., Shibuya, H. and Matsumoto, K. (1999). The TAK1-NLK-MAPK-related pathway antagonizes signaling between beta-catenin and transcription factor TCF. *Nature* **399**, 798-802.

Kaletta, T., Schnabel, H. and Schnabel, R. (1997). Binary specification of the embryonic lineage in *Caenorhabditis elegans*. *Nature* **390**, 294-298.

Krause, M., Fire, A., Harrison, S. W., Priess, J. and Weintraub, H. (1990). CeMyoD accumulation defines the body wall muscle cell fate during *C. elegans* embryogenesis. *Cell* **63**, 907-919.

Krause, M., Harrison, S. W., Xu, S. Q., Chen, L. and Fire, A. (1994). Elements regulating cell- and stage-specific expression of the *C. elegans* MyoD family homolog *hlh-1*. *Dev. Biol.* **166**, 133-148.

Krause, M., Park, M., Zhang, J. M., Yuan, J., Harfe, B., Xu, S. Q., Greenwald, I., Cole, M., Paterson, B. and Fire, A. (1997). A *C. elegans* E/Daughterless bHLH protein marks neuronal but not striated muscle development. *Development* **124**, 2179-2180.

Labouesse, M., Hartwieg, E. and Horvitz, H. R. (1996). The *Caenorhabditis elegans* LIN-26 protein is required to specify and/or maintain all non-neuronal ectodermal cell fates. *Development* **122**, 2579-2588.

Labouesse, M. and Georges-Labouesse, E. (2003). Cell adhesion: Parallels between vertebrate and invertebrate focal adhesions. *Curr. Biol.* **13**, R528-R530.

Lin, R., Thompson, S. and Priess, J. R. (1995). pop-1 encodes and HMG box protein required for the specification of a mesoderm precursor in early *C. elegans* embryos. *Cell* **83**, 599-609.

Lin, R., Hill, R. J. and Priess, J. R. (1998). POP-1 and anterior-posterior fate decisions in *C. elegans* embryos. *Cell* **92**, 229-239.

Liu, J. and Fire, A. (2000). Overlapping roles of two Hox genes and the *exd* ortholog *ceh-20* in diversification of the *C. elegans* postembryonic mesoderm. *Development* **127**, 5179-5190.

Lo, M.-C., Gay, F., Odom, R., Shi, Y. and Lin, R. (2004). Phosphorylation by the beta-Catenin/MAPK complex promotes 14-3-3-mediated nuclear export of TCF/POP-1 in signal-responsive cells in *C. elegans*. *Cell* **117**, 95-106.

Maduro, M. F. and Rothman, J. H. (2002). Making worm guts: the gene regulatory network of the Caenorhabditis elegans endoderm. *Dev. Biol.* **246**, 68-85.

Maduro, M. F., Meneghini, M. D., Bowerman, B., Broitman-Maduro, G. and Rothman, J. H. (2001). Restriction of mesendoderm to a single blastomere by the combined action of SKN-1 and a GSK-3beta homolog is mediated by MED-1 and -2 in *C. elegans*. *Mol. Cell* **7**, 475-485.

McKinsey, T. A., Zhang, C. L. and Olson, E. N. (2001). Control of muscle development by dueling HATs and HDACs. *Curr. Opin. Genet. Dev.* **11**, 497-504.

Mello, C. and Fire, A. (1995). DNA transformation. In *Methods in Cell Biology. Caenorhabditis elegans: Modern Biological Analysis of an Organism.* Vol. 48 (ed. H. F. Epstein and D. C. Shakes), pp. 451-482. San Diego: Academic Press Inc.

Mello, C. C., Draper, B. W., Krause, M., Weintraub, H. and Priess, J. R. (1992). The *pie-1* and *mex-1* genes and maternal control of blastomere identity in early *C. elegans* embryos. *Cell* **70**, 163-176.

Meneghini, M. D., Ishitani, T., Carter, J. C., Hisamoto, N., Ninomiya-Tsuji, J., Thorpe, C. J., Hamill, D. R., Matsumoto, K. and Bowerman, B. (1999). MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in *Caenorhabditis elegans*. *Nature* **399**, 793-797.

Mercer, K. B., Flaherty, D. B., Miller, R. K., Qadota, H., Tinley, T. L., Moerman, D. G. and Benian, G. M. (2003). *Caenorhabditis elegans* UNC-98, a C2H2 Zn finger protein, is a novel partner of UNC-97/PINCH in muscle adhesion complexes. *Mol. Bol. Cell* **14**, 2492-2507.

Michelson, A. M., Abmayr, S. M., Bate, M., Arias, A. M. and Maniatis, T. (1990). Expression of a MyoD family member prefigures muscle pattern in Drosophila embryos. *Genes Dev.* **12**, 2086-2097.

Mickey, K. M. (2000). *Cell-Cell Interactions and the Specification of Cell Fates During C. elegans Embryogenesis.* Thesis, University of Washington, Seattle.

Miller, D. M., Stockdale, F. E. and Karn, J. (1986). Immunological identification of the genes encoding the four myosin heavy chain isoforms of *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **83**, 2305-2309.

Miner, J. H., Miller, J. B. and Wold, B. J. (1992). Skeletal muscle phenotypes initiated by ectopic MyoD in transgenic mouse heart. *Development* **114**, 853-860.

Munsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P. and Lassar, A. B. (1995). Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev*. **9**, 2911-2922.

Polesskaya, A., Seale, P. and Rudnicki, M. A. (2003). Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. *Cell* **113**, 841-852.

Pownall, M. E., Gustafsson, M. K. and Emerson, C. P., Jr (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu. Rev. Cell Dev. Biol.* **18**, 747-783.

Priess, J. R. and Thomson, J. N. (1987). Cellular interaction in early *C. elegans* embryos. *Cell* **48**, 241-250.
Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y. H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**, 707-716.
Rocheleau, C. E., Yasuda, J., Shin, T. H., Lin, R., Sawa, H., Okano, H., Priess, J. R., Davis, R. J. and Mello, C. C. (1999). WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in *C. elegans*. *Cell* **97**, 717-726.
Rogalski, T. M., Mullen, G. P., Bush, J. A., Gilchrist, E. J. and Moerman, D. G. (2001). UNC-52/Perlecan isoform diversity and function in *Caenorhabditis elegans*. *Biochem. Soc. Trans.* **29**, 171-176.
Sabourin, L. A. and Rudnicki, M. A. (2000). The molecular regulation of myogenesis. *Clin. Genet.* **57**, 16-25.
Schnabel, R. (1995). Duels without obvious sense: counteracting inductions involved in body wall muscle development in the *Caenorhabditis elegans* embryo. *Development* **121**, 2219-2232.
Schnabel, R., Weiger, C., Hutter, H., Feichtinger, R. and Schnabel, H. (1996). *mex-1* and the general partitioning of cell fate in the early *C. elegans* embryo. *Mech. Dev*. **54**, 133-147.
Shin, T. H., Yasuda, J., Rocheleau, C. E., Lin, R., Soto, M., Bei, Y., Davis, R. J. and Mello C. C. (1999). MOM-4, a MAP kinase kinase kinase-related protein, activates WRM-1/LIT-1 kinase to transduce anterior/posterior polarity signals in *C. elegans*. *Mol. Cell* **4**, 275-280.
Stringham, E. G., Dixon, D. K., Jones, D. and Candido, E. P. (1992). Temporal and spatial expression patterns of the small heat shock (*hsp16*) genes in transgenic *Caenorhabditis elegans*. *Mol. Biol. Cell* **3**, 221-233.
Strome, S. and Wood, W. B. (1983). Generation of asymmetry and segregation of germ-line granules in early *C. elegans* embryos. *Cell* **35**, 15-25.
Suh, E., Chen, L., Taylor, J. and Traber, P. G. (1994). A homeodomain protein related to *caudal* regulates intestine-specific gene transcription. *Mol. Cell. Biol*. **14**, 7340-7351.
Sulston, J. and Horvitz, H. R. (1977). Post-embryonic cell lineage of *Caenorhabditis elegans*. *Dev. Biol.* **56**, 110-156.
Sulston, J., Schierenberg, E., White, J. and Thomson, J. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64-119.
Tajbakhsh, S. (2003). Stem cells to tissue: molecular, cellular and anatomical heterogeneity in skeletal muscle. *Curr. Opin. Genet. Dev.* **13**, 413-422.
Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M. and Cossu, G. (1998). Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. *Development* **125**, 4155-4162.
Thorpe, C. J., Schlesinger, A., Carter, J. C. and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. *Cell* **90**, 695-705.
Timmons, L., Court, D. L. and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans. Gene* **263**, 103-112.
Waterston, R. H. (1988). Muscle. In *The Nematode* Caenorhabditis elegans. (ed. W. B. Wood). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Wei, Q. and Paterson, B. M. (2001). Regulation of MyoD function in the dividing myoblast. *FEBS Lett.* **490**, 171-178.
Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75**, 1241-1244.
Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S. et al. (1991). The *myoD* gene family: nodal point during specification of the muscle cell lineage. *Science* **251**, 761-766.
Xu, F., Li, H. and Jin, T. (1999). Cell type-specific autoregulation of the *caudal*-related homeobox gene *cdx-2/3*. *J. Biol. Chem.* **274**, 34310-34316.
Zhu, J., Fukushige, T., McGhee, J. D. and Rothman, J. H. (1998). Reprogramming of early embryonic blastomeres into endodermal progenitors by a *Caenorhabditis elegans* GATA factor. *Genes Dev.* **12**, 3809-3814.