The proper formation of a complex multicellular organism requires the precise coordination of many cellular events, including cell proliferation, cell fate specification and differentiation. The *C. elegans* postembryonic mesodermal lineage, the M lineage, allows us to study mechanisms coordinating these events at single cell resolution. We have identified an HMX homeodomain protein MLS-2 in a screen for factors required for M lineage patterning. The MLS-2 protein is present in nuclei of undifferentiated cells in the early M lineage and in a subset of head neurons. In the M lineage, MLS-2 activity appears to be tightly regulated at the fourth round of cell division, coincident with the transition from proliferation to differentiation. A predicted null allele of *mls-2*, cc615, causes reduced cell proliferation in the M lineage, whereas a semi-dominant, gain-of-function allele, *tm252*, results in increased cell proliferation. Loss or overexpression of *mls-2* also affects cleavage orientation and cell fate specification in the M lineage. We show that the increased cell proliferation in *mls-2(tm252)* mutants requires CYE-1, a G1 cell cycle regulator. Furthermore, the *C. elegans* Myod homolog HLH-1 acts downstream of *mls-2* to specify M-derived coelomocyte cell fates. Thus MLS-2 functions in a cell type-specific manner to regulate both cell proliferation and cell fate specification.

**Key words:** *mls-2*, HMX, Nkx5, Homeodomain, *C. elegans*, Mesoderm, Cleavage orientation, Cell proliferation, Cell fate specification, HLH-1, Myod, CYE-1
Materials and methods

C. elegans strains

Strains were manipulated under standard conditions, as described by Brenner (Brenner, 1974). Analyses were performed at 20°C, unless otherwise noted. The following strain was generated for observing the M lineage in cc615 mutant animals throughout postembryonic development: LW0050 [ccls4438(intrinsic CC::gfp) III; asIs2(egl-15::gfp) IV; mls-2(cc615) ayIs6(hlh-8::gfp)] X. The strain LW0081 [ccls4438(intrinsic CC::gfp) III; asIs2(egl-15::gfp) IV; ayIs6(hlh-8::gfp) X] was used as the corresponding wild-type control. Additional cell-type-specific reporters for the M lineage were as described in Kostas and Fire (Kostas and Fire, 2002). The M lineage was followed in live animals under a fluorescence stereomicroscope and confirmed using the compound microscope.

Additional strains used in this work were as follows. LG I: ayIs4 [egl-17::gfp] (Burdine et al., 1998); cye-1(eh10)/dpy-14(e168) (Brodigan et al., 2003); dpy-5(e6) cye-1(ar95)/unc-13(e51) (Seydoux et al., 1993); lin-6(e1466) dpy-5(e6) hth2[qls48(1.III)] (Wang and Kimble, 2001).

LG II: hth-1(cc561ts) (Harfe et al., 1998a).

LG III: mab-5(e1239), lin-39(n760) mab-5(e1239)/dpy-17(e164) unc-32(e189) (Liu and Fire, 2000); pha-1(e2123ts) (Schnabel and Schnabel, 1990).

LG V: him-5(e1467) (Hodgkin et al., 1979).

LG X: hth-8(nr2061) (Cori et al., 2000); unc-2(e55) lon-2(e678), dpy-8(e130) unc-124(hs10) (Hecht et al., 1996); mls-2(tm252) (gift from Shohei Mitani, Tokyo Women’s Medical University School of Medicine, Japan).

Mutagenesis screen and analysis of mls-2

PD4666(ayIs6) animals carrying an integrated hth-8::gfp reporter that labels all undifferentiated M lineage descendants were mutagenized with ethylmethylsulfonate (EMS). Non-clonal F2 populations were screened using a fluorescence stereomicroscope. Animals with abnormal M lineage patterning were recovered for further analysis. We screened 15,000 haploid genomes and isolated 15 independent mutations with an altered M lineage. One recessive mutation, cc615, showed 100% penetrance of M lineage defects and defines the mls-2 locus. Three-factor mapping using unc-2(e55) lon-2(e678) and dpy-8(e130) unc-124(hs10), coupled with snip-SNP mapping (Wicks et al., 2001), placed mls-2 between C07A12.2 and F43C9.2 on the X chromosome. Among 13 likely candidates of the 40 predicted genes located in this region, RNAi of C39E6.4 in wild-type animals phenocopied the cc615 M lineage phenotype. Cosmid C39E6 rescued the cc615 mutant phenotype. The molecular lesions of cc615 were identified through direct sequencing of PCR fragments encompassing the entire coding region of C39E6.4.

Plasmid constructs and transgenic lines

mls-2 reporter constructs

Forced expression constructs

Detailed information on all the constructs is available upon request. Plasmid pKM1110 (cye-1::gfp) was from Mike Krause (Brodigan et al., 2003). Transgenic lines were generated using the plasmid pRF4 (Mello et al., 1991) or the pha-1 rescuing plasmid pC1 (Granato et al., 1994) as markers.
Heat-shock experiments
To examine the effects of forced expression of mls-2 on the M lineage, animals carrying pYJ62[hisp16p::mls-2::mls-2 3’ UTR] were stage synchronized with respect to M lineage development using the integrated hlh-8::gfp. Animals of the same stage were transferred to the same plate, heat shocked at 37°C for 1 hour and analyzed at different time points after recovery at 20°C. The M lineage phenotype was examined using hlh-8::gfp, egl-15::gfp and CC::gfp markers, and compared with that of control animals (LW0081) that had undergone the same treatment.

To examine the consequences of forced expression of mls-2 on other cell types, animals carrying pYJ62 were stage synchronized and heat shocked at 37°C for 30 minutes to 1 hour, every 8 or 12 hours starting from late embryogenesis. The intestinal cells, seam cells and cells in the vulva were examined using cye-1::gfp, egl-17::gfp, DIC optics and DAPI staining.

RNAi
Plasmids yk2769 (gift from Yuji Kohara, National Institute of Genetics, Japan) and pVZ1200 (gift from Mike Krause, NIDDK, NIH, USA) were used as templates for synthesizing dsRNA against mls-2 and hlh-1, respectively, following the protocol of Fire and colleagues (Fire et al., 1998). dsRNA was injected into gravid adults of different genotypes. Progeny of injected animals were scored for larval lethality and M lineage defects. Water-injected animals were used as controls.

Antibodies and immunofluorescence staining
The N terminus of MLS-2 (amino acids 14 to 215) was cloned into pGEX-4T-1 (Smith and Johnson, 1988). Plasmid pYJ67 was transformed into BL21(DE3)pLyS5 cells. Fusion proteins were first purified under denaturing conditions using Glutathione sepharose 4B beads (Amersham Biosciences) and further purified by SDS-PAGE. Gel slices containing the purified fusion proteins were used to immunize rats (Cocalico Biologicals, PA). The resulting antisera were tested by western blot analyses using bacterially generated GST-MLS-2 fusion proteins. Antibodies were further purified by pre-adsorption with extracts of mls-2(cc615) mutant worms at 4°C overnight (Malof and Kenyon, 1998).

For immunostaining using anti-HLH-1 antibodies, animals were fixed following the protocol of Harfe and colleagues (Harfe et al., 1998a). For all other antibodies, animals were fixed following the protocol of Hurd and Kemphues (Hurd and Kemphues, 2003). The following antibodies were used: preadsorbed rat anti-MLS-2 (CUMC-R6; 1:400 to 1:1000), goat anti-GFP (Rockland Immunocoucheous; 1:5000), mouse anti-β-galactosidase (Promega; 1:50) and rabbit anti-HLH-1 (Krause et al., 1990) (gift of M. Krause; 1:400). All secondary antibodies were from Jackson ImmunoResearch Laboratories and were used at a dilution of 1:100 to 1:400. Differential interference contrast and epifluorescence microscopy were performed using a Leica DMRA2 compound microscope. Images were captured by a Hamamatsu Orca-ER camera using the Openlab software (version 3.0.9, Improvisation). Subsequent image analysis was performed using Adobe Photoshop 7.0.

Results
**mls-2(cc615) mutants display abnormal cleavage orientation, under-proliferation and cell fate transformation in the M lineage**
In a screen for mutants with abnormal M lineage patterning (see Materials and methods), we isolated the mutation cc615. Animals homozygous for cc615 were 100% penetrant for their M lineage defects, and exhibited approximately 30% larval and adult lethality. The mutation was completely recessive and showed no evidence of defects from maternal inheritance. We designated the gene corresponding to cc615 mls-2 (*mesodermal lineage specification-2*).

We analyzed the M lineage in cc615 mutants by following post-embryonic development in live animals using molecular markers that label different cell types of the M lineage. Using the hlh-8::gfp reporter as a marker for undifferentiated cells in the M lineage (Harfe et al., 1998b), the first defect we observed was abnormal cleavage orientation in cc615 mutants. In wild-type animals, the M mesoblast always undergoes the first cell division along the dorsoventral axis (Fig. 2A). However, among the 32 cc615 mutants examined, only 69% divided with this orientation, and the cleavage plane was sometimes tilted (data not shown). In the remaining 31%, the first division of M was oriented in the anteroposterior direction (Fig. 2B). Very rarely, we observed M division along the left-right axis (data not shown). Subsequent cleavage orientations of the M lineage divisions continued to be randomized in cc615 mutants (Fig. 3B-D). The resulting M descendants were located in the posterior of cc615 mutant animals in variable patterns (see Fig. 2E as an example), instead of being positioned in four quadrants, as in wild-type animals (Fig. 2D).

In addition to the cleavage orientation defects, cc615 mutants also exhibited defects in cell proliferation and cell fate specification in the M lineage. In wild-type hermaphrodites, the M mesoblast first undergoes four rounds of cell division to produce 16 descendants expressing hlh-8::gfp (Fig. 1). In cc615 mutant animals, M always completed the first three rounds of cell divisions, generating eight hlh-8::gfp expressing cells. However, zero to six of the eight cells divided for a fourth time in cc615 mutants, yielding a range of 8-14 M lineage descendants (Fig. 2E, Fig. 3B-D). Thus at late L1 stage, mls-2(cc615) mutant animals contained a reduced number of M lineage descendants when compared with wild-type animals at the same stage.

After the fourth round of cell division in the wild-type M lineage, two of the 16 cells on the ventral side undergo an extra round of division, with the M lineage producing 14 BWMs, 2 CCs and 2 SMs (Fig. 1). Using Nomarski optics in combination with the myo-3::gfp marker and the intrinsic cc::gfp marker, which label BWMs and CCs, respectively (Kostas and Fire, 2002), we found that all cc615 mutants had the correct number of embryonically derived CCs and BWMs, but lacked M-derived CCs, and most or all M-derived BWMs (Table 1, Fig. 2J,K, Fig. 3B-D). Instead, cc615 mutants contained more than two M-derived cells (three to nine cells) that behaved like SMs (Table 1, Fig. 3B-D). These SM-like cells were enlarged, expressed hlh-8::gfp and migrated toward the vulva to various degrees (Fig. 2H). They divided two to three times to generate multiple sex muscle precursors that differentiated into appropriate vulval and uterine muscles, as verified by a number of sex muscle-specific GFP markers, including egl-15::gfp, arg-1::gfp and NdE box::gfp (Fig. 2K).

The presence of vulval muscles was also confirmed by observing the light diffraction of the muscles using polarized light. Among the SM-like cells in cc615 mutants, two cells always migrated to the ventral side around the vulva and generated the correct number of sex muscles that attached properly and appeared functional. cc615 mutant animals did not have any egg-laying defects (data not shown). Thus, cc615 mutant animals produce extra SMs at the expense of M lineage-derived CCs and BWMs.
mls-2 encodes a homeodomain protein of the HMX family

We mapped mls-2 to an interval between C07A12.2 and F43C9.2 on the left arm of chromosome X. This region covers 40 predicted genes. RNAi injection of one of them, C39E6.4, resulted in close to 100% penetrance of M lineage defects similar to those observed in mls-2(cc615) mutants, suggesting that C39E6.4 was mls-2 (see Materials and methods). We rescued the cc615 mutant phenotypes with a DNA fragment containing the genomic region of C39E6.4 and identified two molecular lesions in the coding region of C39E6.4 in cc615 mutants (Fig. 4A). The first lesion is a nonsense mutation at residue 28 (CAA to TAA, Gln to Ochre Stop) and the second lesion is a mis-sense mutation at residue 55 (GAG to GAT, Glu to Asp). The presence of the premature stop codon suggests that cc615 is likely a null allele. Additional evidence that cc615 is a null mutation is provided by the immunostaining results discussed below (Fig. 4A).

The predicted MLS-2 protein is 341 amino acids in length and contains a homeodomain between residues 201 and 260 (Fig. 4A,B). This homeodomain is most similar to that of the Nkx5 family of homeodomain proteins, also known as the HMX family. The HMX gene family is present in many organisms from sea urchins to humans (Adamska et al., 2000; Adamska et al., 2001; Bober et al., 1994; Deitcher et al., 1994; Herbrand et al., 1998; Martinez and Davidson, 1997; Rinkwitz-Brandt et al., 1995; Rinkwitz-Brandt et al., 1996; Shaw et al., 2003; Stadler et al., 1992; Stadler et al., 1995; Stadler and Solursh, 1994; Wang et al., 1990; Wang et al., 2000; Yoshiura et al., 1998). HMX proteins share homology in the
showed identical expression patterns (Fig. 5A-H). Expression of MLS-2 was first detectable in one or two cells in embryos at the 50-cell stage (data not shown) and is localized in the nucleus. MLS-2 continued to be expressed in proliferating cells that are primarily located at the anterior of the embryo and are presumably derived from the AB lineage (Fig. 5A-D). During morphogenesis, this expression became restricted to a small subset of head neuronal precursors. Expression persisted in six head neurons during postembryonic development (Fig. 5E,F). We also observed GFP::MLS-2 expression in unidentified cells near the vulva at the L2 and L3 stages (data not shown).

To characterize the M lineage expression pattern of mls-2, we performed double-labeling experiments using anti-MLS-2 antibodies and the M lineage-specific hlh-8::gfp or hlh-8::lacZ markers (Harfe et al., 1998b). mls-2 expression in the M lineage was first detectable in the M mesoblast (Fig. 5P-R), and was retained during the first three rounds of cell divisions, such that mls-2 expression was still detectable in eight M descendants (designated 8-M stage, n=200, Fig. 5M,N). However, after one more round of cell division (at the 16-M stage), no MLS-2 signal was detected either by anti-MLS-2 antibodies or by the gfp::mls-2 fusion construct (n>50, 4123

Table 1. Differentiated M lineage descendants in mls-2 and cye-1 mutant animals

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of M-derived BWMs (myo-3::gfp)</th>
<th>Number of M-derived CCs (intrinsic CC::gfp)</th>
<th>Number of SMs (hlh-8::gfp)</th>
<th>Number of VM1s (egl-15::gfp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>14 (n=3)</td>
<td>2 (100%) n&gt;100</td>
<td>2 (n=100)</td>
<td>4 (n&gt;100)</td>
</tr>
<tr>
<td>cc615/cc615</td>
<td>0-3 (n=16)</td>
<td>0 (100%) n&gt;100</td>
<td>3-9 (n=32)</td>
<td>6-14 (n=50)</td>
</tr>
<tr>
<td>tm252/tm252*</td>
<td>17-25 (n=6)</td>
<td>0 (32%) 1-2 (51%) n=115 3-4 (17%)</td>
<td>2-9 (n=29)</td>
<td>4-12 (n=43)</td>
</tr>
<tr>
<td>tm252+/+</td>
<td>N/A</td>
<td>0 (48%) 1-2 (47%) n=62 3 (5%)</td>
<td>2-5 (n=21)</td>
<td>4-8 (n=62)</td>
</tr>
<tr>
<td>eh10/eh10</td>
<td>N/A</td>
<td>0 (19%) 1 (81%) n=63</td>
<td>1-3 (n=100)</td>
<td>2-5 (n&gt;100)</td>
</tr>
<tr>
<td>eh10/eh10; tm252/ tm252</td>
<td>N/A</td>
<td>0 (90%) 1 (10%) n=85</td>
<td>1-3 (n=100)</td>
<td>2-5 (n&gt;100)</td>
</tr>
</tbody>
</table>

*4% of tm252/tm252 animals (n=115) and 18% of tm252/+ animals (n=62) were completely wild type with regard to the M lineage.

†Some egl-15::gfp-positive cells lacked the morphology of normal vulval muscles.
Fig. 4. *mls-2* encodes a homeodomain protein of the HMX family. (A) *mls-2* gene structure and constructs. The upper diagram represents the six exons of the *mls-2* genomic sequence, with the molecular lesions in *cc615* and *tm252* indicated. Shaded areas represent the homeodomain. The lower diagram depicts various fusion constructs used in this work (see Materials and methods). Both pYJ59 and pYJ62 were able to rescue the *mls-2(cc615)* mutant phenotypes. (B) A Clustal W sequence alignment of MLS-2, other HMX (Nkx5) family homeodomain proteins and the *C. elegans* Nkx2.5 homolog CEH-22. Only residues within the homeodomain (residues 1-60) and two additional domains (in dashed boxes) immediately after the homeodomain were aligned. The proteins share no similarity outside this compared region. Identical amino acids are in white letters against black background. Similar amino acids are in black letters against gray background. The alignment is shown for CeMLS-2 (this work), *C. briggsae* CBG14538 (WormBase), SOHo1 (chicken) (Deitcher et al., 1994), SpHmx (sea urchin) (Martinez and Davidson, 1997), TgHbox5 (sea urchin) (Wang et al., 1990), GH6 (chicken) (Stadler et al., 1994), DHmx (*Drosophila*) (Wang et al., 2000), murine Hmx3 (Nkx-5.1) (Bober et al., 1994), murine Hmx2 (Nkx-5.2) (Bober et al., 1994), human HMX2 (Wang et al., 2000), murine Hmx1 (Yoshiura et al., 1998), human HMX1 (H6) (Stadler et al., 1992) and CEH-22 (Okkema and Fire, 1994). (C) A phylogenetic tree of the HMX family generated using the neighbor-joining method of Clustal W. The tree is rooted with CEH-22 in TreeView version 1.6.6 (Page, 1996). Only the homeodomain and the two additional conserved domains immediately after the homeodomain (as shown in B) were used in the analysis.
data not shown). Although we cannot rule out the possibility that a low level of MLS-2 protein is present after the 8-M stage, the loss of MLS-2 signal at the 16-M stage appears to be due to the instability of the MLS-2 protein, because the mls-2 promoter is still active in M lineage descendants after the fourth round of cell division, as detected by a transcriptional mls-2p::gfp::mls-2 3’ UTR construct (Fig. 5O). Neither the mls-2 promoter activity nor the MLS-2 protein was detected in the SM lineage (Fig. 5S-U) or the differentiated BWMs and CCs (data not shown). Thus mls-2 is expressed in the proliferating cells of the early M lineage (Fig. 5V).

Fig. 5. Expression pattern of mls-2 in wild-type, cc615 and tm252 hermaphrodites. (A,B,E,F,M) Wild-type embryos (A,B) and L1 larvae (E,F,M) stained with anti-MLS-2 antibody (A,E,M) or DAPI (B,F). (C,G,N) Expression pattern of a functional GFP::MLS-2 fusion in wild-type embryos (C) and early L1 larvae (G,N). (D,H) Corresponding DIC images for panels C and G. Nuclear localized MLS-2 protein was seen in a subset of cells during embryogenesis (A,C), in six head neurons (arrowhead in E; the neuronal signal was out of focus in G), in the M mesoblast (arrows in E and G) and in the eight M descendants (M,N; only four cells visible in this focal plane). No MLS-2 protein was detected after the 8-M stage (n>50). Note that the other signals shown in G are due to gut autofluorescence. (I-L) cc615 (I,J) and tm252 (K,L) L1 larvae stained with anti-MLS-2 antibody (I,K) or DAPI (J,L). (I) No MLS-2 staining was detected in cc615 mutants (n>50). (K) The MLS-2(gf) protein in tm252 mutants was detected in the 16 M descendants (eight visible in this focal plane) and the head neurons (data not shown). (O) GFP signal from a mls-2p::gfp transgene in an animal with 13 M descendants (six GFP-expressing cells visible in the focal plane shown). The GFP signal is primarily cytoplasmic. (P-U) Transgenic animals carrying an integrated hlh-8p::lacZ reporter were double-stained with both anti-MLS-2 (PS) and anti-β-galactosidase (QT) antibodies. (R,U) Merged images, MLS-2 protein was detected in the M mesoblast (P-R), but not in the SMs (S-U). (V) Summary of mls-2 expression pattern in the M lineage. The wild-type M lineage with overlay of mls-2 expression highlighted in red. Solid red lines indicate mls-2 expression detected by both GFP::MLS-2 and anti-MLS-2 antibodies; dashed red lines indicate mls-2 expression detected only by a transcriptional mls-2p::gfp reporter.
A gain-of-function mls-2 mutation, tm252, causes over-proliferation and abnormal cell fate specification in the M lineage

A deletion allele of mls-2, tm252, was generated by Shohei Mitani’s laboratory. tm252 contains a deletion of 480 bp of the mls-2 genomic sequences (from the middle of intron 2 to the beginning portion of exon 4) and an insertion of a T after the deletion (Fig. 4A). We out-crossed the tm252 mutants and analyzed their M lineage phenotypes. Unlike cc615, tm252 did not affect cleavage orientation in the M lineage (Fig. 2C). However, extra cell divisions occurred after four rounds of proper cell division in more than 80% of tm252 mutant animals scored (n>50), resulting in more than 18 hblh-8::gfp expressing cells in the early M lineage (see Fig. 2F for an example). We also observed extra M-derived BWMs and SMs, as well as variable numbers of CCs in tm252 mutants (Fig. 2I, Table 1). Because some tm252 animals had no M-derived CCs (32%, n=115), it is possible that the tm252 mutation also causes cell fate transformation in the M lineage.

In order to gain insight into the nature of the tm252 mutation, we examined the localization of the mutant MLS-2 protein in tm252 animals. Antibody staining showed that the mutant MLS-2 protein in tm252 animals was stably made in both the M lineage (Fig. 5K,L) and the head neurons (data not shown), identical to that of wild-type MLS-2 protein. However, the mutant MLS-2 protein perdured longer and was still detectable at the 16-M stage in tm252 mutants (Fig. 5K,L), unlike wild-type MLS-2, which is absent at this stage. Perdurance of the mutant protein was also detected in head neurons in tm252 larvae (data not shown). This perdurance appeared to be transient, as we did not detect any mutant MLS-2 protein in the SM lineage and the signal in the additional neurons eventually faded away at the L2 larval stage (data not shown).

Several lines of evidence showed that tm252 behaved as a semi-dominant, gain-of-function allele. First, tm252/+ animals exhibited similar, although less severe, M lineage defects compared with tm252/tm252 animals (Table 1). Second, as described above, the mutant MLS-2 protein in tm252 animals perdured longer after the 8-M stage in the M lineage (Fig. 5K,L). Finally, removal of the mutant MLS-2 protein in tm252 animals by mls-2(RNAi) led to abnormal division orientations, reduced cell proliferation and complete loss of CC fates, phenocopying the M lineage phenotypes of cc615 mutants.

Misc- and overexpression of mls-2 cause a variety of defects in the M lineage

Since loss or gain of mls-2 function caused proliferation defects in the M lineage, we next examined the consequences of misc- and overexpression of mls-2 inside and outside of the M lineage. We introduced a heat-shock inducible hsp::mls-2 transgene (pYJ62) into the strain LW0081 (see Materials and methods). We then applied heat shock at various stages of larval development and examined the consequences. As expected, anti-MLS-2 antibody staining revealed high levels and global expression of MLS-2 in these transgenic animals after heat shock (data not shown).

Endogenous mls-2 expression was detected in the M lineage from 1-M to 8-M stages. When heat shocked during these stages, a small fraction of transgenic animals died as larvae (data not shown). The remaining animals developed to fertile adults, but with various M lineage defects, including randomized cleavage orientations, increased cell proliferation and abnormal numbers of CCs and SMs (Table 2). Thus, the level of mls-2 gene product in the M lineage is critical for its proper development.

Endogenous mls-2 expression was not detected in the SMs or their descendants. When transgenic animals were heat shocked during 2-SM (L2 stage) to 16-SM (L3 stage) stages, CC fates were not affected. By contrast, the undifferentiated SM-descendants underwent extra rounds of cell divisions, resulting in extra numbers of VM1s (Table 2). Thus, mls-2 is sufficient to promote cell proliferation when ectopically expressed in the SM lineage.

All M lineage-derived cells are fully differentiated at late L4 and adult stages. When heat shock was applied to transgenic animals at these stages, no M lineage defects were observed, suggesting that forced expression of mls-2 in differentiated cells of the M lineage had no effect.

We also subjected the transgenic animals to multiple rounds of heat-shock during larval development and examined other cell types outside of the M lineage, including intestinal cells, seam cells and cells in the vulva (see Materials and methods). Although we observed slower growth and a 10% penetrance of the Pvl phenotype in the transgenic animals, we found no evidence of increased cell proliferation in the cell types examined (data not shown).

Table 2. Effect of forced expression of MLS-2 on the M lineage

<table>
<thead>
<tr>
<th>Phenotypes (number of M-derived CCs and VM1s)</th>
<th>Overexpressing mls-2 in the early M lineage</th>
<th>Ectopically expressing mls-2 in the SM lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type control (n=65)</td>
<td>hsp::mls-2 (n=35)</td>
<td>Wild-type control (n=35)</td>
</tr>
<tr>
<td>0 CCs, &gt;4 VM1s</td>
<td>0</td>
<td>23%</td>
</tr>
<tr>
<td>1 CCs, &gt;4 VM1s</td>
<td>0</td>
<td>9%</td>
</tr>
<tr>
<td>2 CCs, 4 VM1s</td>
<td>100%</td>
<td>14%</td>
</tr>
<tr>
<td>2 CCs, &gt;4 VM1s</td>
<td>0</td>
<td>28%</td>
</tr>
<tr>
<td>3-5 CCs, &gt;4 VM1s</td>
<td>0</td>
<td>26%</td>
</tr>
</tbody>
</table>

mls-2(tm252) requires cye-1 to promote extra cell divisions in the M lineage

To better understand the mechanism of how MLS-2 regulates cell proliferation in the M lineage, we looked for cell cycle regulators that might be involved in the hyper-proliferation in tm252 mutants. One cell cycle regulator is CYE-1, a primary trigger of S phase (Moroy and Geisen, 2004; Fay and Han, 2000; Brodigan et al., 2003).

There are two strong loss-of-function alleles of cye-1, ar95 and eh10 (Fay and Han, 2000; Brodigan et al., 2003). We found that both alleles showed similar M lineage defects: a subset of cells generated from the first three rounds of cell divisions
failed to divide for a fourth time, and one or both M-derived CCs were missing (Table 1). Similar to the findings of Brodigan et al. (Brodigan et al., 2003), most cye-1 mutant animals contained two SMs (~95%, n>100), and the SMs exhibited variable division patterns ranging from no division to the wild-type number of divisions. A subset of the SM descendants differentiated into cells expressing egl-15::gfp (Table 1).

Because both cye-1(eh10) and mls-2(cc615) mutants exhibited reduced proliferation during the fourth round of cell division in the M lineage, we investigated the relationship between cye-1 and mls-2. Using a cye-1::gfp reporter (Brodigan et al., 2003), we observed cye-1::gfp expression in wild-type, cc615 and tm252 animals throughout the early M lineage, suggesting that mls-2 does not regulate the zygotic transcription of cye-1. However, lack of CYE-1 activity suppressed the hyperplasia of tm252 mutants in the M lineage: cye-1(eh10); mls-2(tm252) double mutants failed to divide excessively and had one or two M-derived CCs, resembling the cye-1(eh10) single mutants (Table 1). This indicates that CYE-1 activity is required for the hyper-proliferative defects associated with tm252.

**HLH-1 is a downstream target of MLS-2 in regulating cell fate specification**

In addition to reduced cell proliferation, cc615 mutants exhibited randomized cleavage orientation and cell fate transformation from CCs to BWMS to SMs in the M lineage. Several previously described mutants share similar M lineage defects. mab-5(e1239) and hlh-8(nr2061) mutants show abnormal cleavage orientation in the M lineage, whereas mab-5(e1239) and hlh-1(cc561ts) exhibit fate transformation from CCs to SMs (Kenyon, 1986; Harfe et al., 1998a; Harfe et al., 1998b; Corsi et al., 2000; Corsi et al., 2002). Both mab-5 (encoding a Hox factor) and hlh-8 (encoding CeTwist) are expressed in the early M lineage, with hlh-8 being a direct target of mab-5 and lin-39 (S. J. Salser, PhD thesis, University of California, San Francisco, 1995) (Harfe et al., 1998b; Liu and Fire, 2000). hlh-1 encodes CeMyoD and is expressed in both the embryonic and M-derived BWMS, as well as in the early M lineage from the 2-M stage to 16-M stage, prior to differentiation (Krause et al., 1990; Harfe et al., 1998a).

We investigated the regulatory relationship between mls-2 and mab-5, and hlh-8 and hlh-1. Normal levels and patterns of MLS-2 expression were detected by anti-MLS-2 antibodies in lin-39(n1760) mab-5(e1239), hlh-8(nr2061) and hlh-1(cc561ts) mutants at the non-permissive temperature (Fig. 6A,B, data not shown). Similarly, expression of mab-5::gfp and hlh-8::gfp was detected in the M lineage of cc615 mutants (Fig. 2B-H, Fig. 6C). By contrast, the M lineage-specific expression of HLH-1 was not detectable by anti-HLH-1 antibodies in cc615 mutants, even though the same animals showed positive staining of HLH-1 in the embryonic BWMS (Fig. 6D-F). These results suggest that the M lineage expression of hlh-1 requires MLS-2 activity. Consistently with this, hlh-1(RNAi) in cc615 mutants did not produce any additional phenotypes in the M lineage. Furthermore, hlh-1(cc561ts); mls-2(tm252) double mutants completely lacked M-derived CCs (data not shown), resembling the phenotype of hlh-1(cc561ts) single mutants. Additional cell proliferation in the M lineage was still detected in the hlh-1(cc561ts); mls-2(tm252) double mutants, indicating that HLH-1 is not required for MLS-2 function in cell proliferation. Thus MLS-2 acts through HLH-1 to regulate coelomocyte fate specification in the M lineage. Forced expression of hlh-1 in the M lineage could not rescue the loss of CC fates in cc615 mutants (data not shown), suggesting that at least one other target of MLS-2 exists in regulating CC fate specification in the M lineage.

**Discussion**

**MLS-2 is both necessary and sufficient to promote cell proliferation in the M lineage**

We have shown that loss of MLS-2 function in cc615 mutants resulted in reduced proliferation during the fourth round of cell division, whereas a gain-of-function allele, tm252, caused increased proliferation in the M lineage (Fig. 2). In addition,
forced expression of mls-2 in wild-type animals, using either the hlh-8 promoter (data not shown) or the heat-shock promoter (Table 2), resulted in over-proliferation in the M lineage. Thus MLS-2 is both necessary and sufficient for promoting cell proliferation in the M lineage. The ability of MLS-2 to promote cell proliferation appears to be limited to the M lineage. Although forced expression of mls-2 caused over-proliferation in the early M lineage and the SM lineage, we did not observe any over-proliferation in the examined cell types outside of the M lineage. This suggests that other cofactor(s) specifically expressed in the M lineage might be required for MLS-2 to function in regulating cell proliferation.

Previous studies have shown that the G1 cyclin CYE-1 is present in many postembryonic blast cells, and is required for vulval and M lineage development (Fay and Han, 2000; Brodigan et al., 2003). Fay and Han (Fay and Han, 2000) have also reported that only the last rounds of cell divisions during vulval development are affected in cye-1 mutants. Consistent with their findings, we observed that cye-1(eh10) mutants exhibited proliferation defects only after the first three rounds of cell divisions in the M lineage, suggesting that maternal CYE-1 is sufficient for those cell divisions. Because mls-2(cc615) mutants and cye-1(eh10) mutants showed very similar proliferation defects, MLS-2 may be required to regulate either the expression or the activity/stability of CYE-1. We found that zygotic expression of cye-1 was not affected in cc615 mutants. However, a cye-1 null mutation, cye-1(eh10), suppressed the over-proliferation defects of mls-2(tm252) mutants. Thus MLS-2 might regulate the stability and/or activity of CYE-1 in the M lineage.

**MLS-2 controls cell fate specification through HLH-1 in the M lineage**

In addition to regulating cell proliferation, MLS-2 also plays a role in regulating cell fate specification. mls-2(cc615) mutants exhibit cell fate transformation from BWMS and CCs to SMs, suggesting that MLS-2 function is required for the CC and BWM fates. One of the known factors required for these fates is the C. elegans Myod homolog HLH-1 (Harfe et al., 1998a). Our data suggest that HLH-1 is a specific target of MLS-2 in regulating cell fate specification. We have found that HLH-1 is not present in the M lineage in cc615 mutants (Fig. 6), and that reducing HLH-1 activity in tm252 mutants suppresses the CC cell fate without affecting the hyper-proliferation defects. Currently it is not clear whether hlh-1 is a direct target of MLS-2 and whether other targets of MLS-2 also exist in regulating cell fate specification in the M lineage.

**MLS-2 has multiple and distinct functions in regulating cleavage orientation, cell proliferation and cell fate specification**

MLS-2 is a cell type-specific homeodomain protein that regulates cleavage orientation, cell proliferation and fate specification in the M lineage. Several lines of evidence support the model that MLS-2 exerts its multiple functions through distinct mechanisms. First, the gain-of-function tm252 mutants only exhibited defects in cell proliferation and fate specification without affecting cleavage orientation, suggesting that the function of MLS-2 in regulating cleavage orientation can be uncoupled from its other functions. Second, lack of CYE-1 activity specifically suppressed the hyper-proliferation defects of tm252 mutants, whereas removal of HLH-1 activity specifically affected CC fate specification. These results suggest that MLS-2 has distinct sets of downstream target genes regulating cell proliferation and cell fate specification.

Previous studies in a number of systems have shown that cell fate specification is tightly coupled to cell division and cell cycle progression (Cremisi et al., 2003; Malicki, 2004). In particular, progression through the cell cycle is required for the expression of cell fate determinants, as shown in the fly nervous system (Weigmann and Lehner, 1995) and the C. elegans vulval precursor cells (Ambros, 1999). We failed to detect HLH-1 expression even in proliferating cells in the M lineage of mls-2(cc615) mutants, suggesting that the expression of HLH-1 was not dependent on cell cycle progression in the M lineage. At this point, we cannot rule out the possibility that other cell fate determinant(s) are not expressed in mls-2(cc615) mutants because of reduced cell proliferation.

**Developmental control of MLS-2 activity and coordination between cell proliferation and differentiation**

Our studies suggest that mls-2 expression can be regulated at both the transcriptional level and the post-transcriptional level. At the transcriptional level, the mls-2 promoter is specifically active in the early M lineage (1-M to 16-M stage). At the post-transcriptional level, MLS-2 protein can only be detected up to the 8-M stage (Fig. 5). Interestingly, it is at the 16-M stage when most M-derived cells exit the cell cycle and become differentiated (Fig. 1). Because mutants lacking MLS-2 fail to undergo the fourth round of cell division after the 8-M stage, MLS-2 needs to be present in the eight proliferating M lineage descendants to allow them to divide. Once these cells divide, MLS-2 activity needs to be promptly removed to allow these cells to exit the cell cycle and differentiate. Consistent with this, we observed excessive proliferation in tm252 mutants where the mutant MLS-2 protein per-dured beyond the 8-M stage. Thus MLS-2 appears to be a pro-proliferation and/or anti-differentiation factor whose activity needs to be tightly regulated to allow the transition from cell proliferation to differentiation. Because the tm252 allele deletes the entire exon 3 and part of exon 4, those regions may contain the regulatory element(s) that are required for regulating the stability of the MLS-2 protein.

**The HMX protein family: conserved and divergent sequences and functions**

HMX family of homeodomain proteins share a highly conserved homeodomain and two additional conserved regions located immediately C-terminal to the homeodomain (Fig. 4). These two additional regions do not appear to be conserved in MLS-2 and the chick protein SOHo1 (Deitcher et al., 1994) (Fig. 4). Outside of these conserved regions, sequences are highly diverged among the HMX family members.

Previous studies have shown that the vertebrate and the Drosophila HMX proteins share a common expression in the nervous system, with the vertebrate HMX proteins also being expressed in a number of sensory organs (Adamska et al., 2000; Adamska et al., 2001; Bober et al., 1994; Deitcher et al., 1994; Hadrys et al., 1998; Herbrand et al., 1998; Kierman et al., 1997; Rinkwitz-Brandt et al., 1995; Rinkwitz-Brandt et al.,
We have found that MLS-2 plays crucial roles in the development of the postembryonic mesoderm in *C. elegans*. Expression of HMX proteins outside of the nervous system and the sensory organs has been reported, and mice lacking *Hmx3* show inner ear defects and maternal reproductive defects (Martinez and Davidson, 1997; Shaw et al., 2003; Stadler and Solursh, 1994; Wang et al., 1998). Therefore, like *mls-2*, the *Hmx* genes in other organisms might also be expressed and functioning in additional cell types, especially during postembryonic development.

We have shown that MLS-2 is required for cell proliferation and fate specification in the M lineage. Mice that lack *Hmx2*, or both *Hmx2* and *Hmx3*, exhibit reduced proliferation in the otic epithelium and the adjacent mesenchyme, as well as alteration of cell fates in the otic vesicle (Wang et al., 2001; Wang et al., 2004). Interestingly, although *Drosophila Hmx* can functionally replace *Hmx2* and *Hmx3* in the murine inner ear and hypothalamus, it appears to be more effective in rescuing the decreased cell proliferation defects of the *Hmx2*; *Hmx3* double knockout mice (Wang et al., 2004). These observations suggest a conserved function of the HMX proteins in the nervous system. We have also observed neuronal expression of *mls-2* (Fig. 5). Thus, MLS-2 might share with other HMX family members their conserved functions in the nervous system.

References


C. elegans vulval induction to select the outcome of Ras signaling. Development 125, 181-190.


