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Caenorhabditis elegans Twist plays an essential role in non-striated muscle development

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SUMMARY

The basic helix-loop-helix (bHLH) transcription factor Twist plays a role in mesodermal development in both invertebrates and vertebrates. In an effort to understand the role of the unique *Caenorhabditis elegans* Twist homolog, *hlh-8*, we analyzed mesodermal development in animals with a deletion in the *hlh-8* locus. This deletion was predicted to represent a null allele because the HLH domain is missing and the reading frame for the protein is disrupted. Animals lacking CeTwist function were constipated and egg-laying defective. Both of these defects were rescued in transgenic mutant animals expressing wild-type *hlh-8*. Observing a series of mesoderm-specific markers allowed us to characterize the loss of *hlh-8* function more thoroughly. Our results demonstrate that CeTwist performs an essential role in the proper development of a subset of mesodermal tissues in *C. elegans*. We found that CeTwist was required for the formation of three out of the four non-striated enteric muscles born in the embryo. In contrast, CeTwist was not required for the formation of the embryonically derived striated muscles. Most of the post-embryonic mesoderm develops from a single lineage. CeTwist was necessary for appropriate patterning in this lineage and was required for expression of two downstream target genes, but was not required for the expression of myosin, a marker of differentiation. Our results suggest that mesodermal patterning by Twist is an evolutionarily conserved function.

Key words: CeTwist, *C. elegans*, Mesoderm, *hlh-8*, Enteric muscles, Sex muscles, bHLH

INTRODUCTION

Transcription factors such as basic helix-loop-helix (bHLH) proteins exert their influence on development by causing changes in tissue-specific gene expression. bHLH factors are involved in various aspects of differentiation and determination in developmental processes such as neurogenesis and myogenesis (for several recent reviews see: Jan and Jan, 1993; Firulli and Olson, 1997; Baylies et al., 1998; Taylor, 1998; Roy and VijayRaghavan, 1999). These proteins dimerize through contacts made in the helix-loop-helix domains, and the two basic domains in the dimer bind DNA. Twist homologs comprise a subfamily of bHLH proteins based on amino acid identity and signature amino acids in the bHLH domains. Members of the Twist subfamily have been identified in *Drosophila melanogaster* (Thisse et al., 1987; Thisse et al., 1988; Murre et al., 1989), *Mus musculus* (Wolf et al., 1991), humans (Wang et al., 1997), *Xenopus* (Hopwood et al., 1989), leeches (Soto et al., 1997), lancelets (Yasui et al., 1998), and *C. elegans* (Harfe et al., 1998b). Among several of these organisms, the common theme in Twist function is an influence on patterning and development of tissues derived from the middle embryonic germ layer, the mesoderm. In the *D. melanogaster* embryo, *twist* is required for mesoderm specification (Simpson, 1983; Nüsslein-Volhard et al., 1984). Later in fly development, specific subsets of mesodermal tissues require *twist.* For example, *twist*-dependent tissues include indirect flight muscles but not direct flight muscles (Anant et al., 1998). The *M. musculus twist* homolog, *M-twist*, has a specialized mesodermal function akin to the later requirement in flies. *M-twist* is required in only a subset of mesodermal cells; most prominently, it is required in the head mesenchyme for neural tube closure (Chen and Behringer, 1995).

In *C. elegans*, some mesodermal tissues develop in the embryo and others develop post-embryonically (Sulston and Horvitz, 1977; Sulston et al., 1983; Waterston, 1988; Moerman and Fire, 1997). Most body wall muscles, the enteric and pharyngeal muscles, and four non-muscle cells called coelomocytes develop embryonically (Sulston et al., 1983). During larval development, the remainder of the non-gonadal mesoderm develops from the M blast cell. The M mesodermal descendants include a minority of the body wall muscles, the sex-specific muscles, and two additional coelomocytes (Sulston and Horvitz, 1977). Ablation experiments have shown that none of the cells derived from M are required for worm survival (Sulston and White, 1980).

hlh-8 is the only Twist family member among the 24 bHLH

genes in *C. elegans* (Harfe et al., 1998b; Ruvkin and Hobert, 1998). Polyclonal antibody staining and *gfp* reporter expression has revealed the *hlh-8* gene to be active in a subset of mesodermal nuclei. These include the embryonically born enteric muscles and the cells of the post-embryonic M mesoblast lineage (Harfe et al., 1998b). Similarly, expression of the *M. musculus twist* and later expression of *twist* in *D. melanogaster* is restricted to a subset of mesodermal tissues (Wolf et al., 1991; Füchtbauer, 1995; Gitelman, 1997; Thisse et al., 1988; Bate et al., 1991).

This paper examines the role of CeTwist in mesoderm development. The simplicity of the defined *C. elegans* lineage has allowed us to investigate CeTwist function with single cell resolution. We have characterized the cellular defects in a predicted null mutant and find that CeTwist is required for several different mesodermal events. These include the embryonic formation of the non-striated enteric muscles, the patterning in the post-embryonic M mesodermal lineage, and the later formation of the non-striated sex muscles.

MATERIALS AND METHODS

Mutant isolation and molecular characterization

nr2061 animals were isolated in a PCR-based deletion screen (Liu et al., 1999) and kindly provided to us by Axys Pharmaceuticals (NemaPharm Group, South San Francisco, CA). In order to determine the precise location of the mutation in *hlh-8*, we used single animal PCR (Williams, 1995) with a variety of primers to amplify segments of *hlh-8* genomic DNA. We amplified overlapping segments of *hlh-8* in quadruplicate and sequenced directly from the PCR fragments by thermal cycling with Perkin Elmer (Warrington, WA) dRhodamine Termination mix followed by fragment separation on a Perkin Elmer ABI sequencer.

Rescue of *nr2061* mutant animals with genomic *hlh-8* DNA

Transgenic animals were made by standard injection techniques (Mello and Fire, 1995) using the plasmid pBH64 (a gift of Brian Harfe) at a concentration of 50 μg/ml. The dominant marker *rol-6* (pRF4; Mello et al., 1991) was included as a selectable marker in all injections. pBH64 contains the entire genomic coding region of *hlh-8* and approximately 9 kb upstream and 3 kb downstream of genomic DNA. The plasmid pBH64 includes an uncharacterized open reading frame (ORF) upstream of *hlh-8* (Harfe et al., 1998b). To ensure that gene expression from the *hlh*-8 locus was the source of rescue from pBH64, we filled in a unique *Mlu*I restriction site in exon 1 of *hlh-8* using the Klenow fragment of DNA polymerase (New England Biolabs, Inc., Beverly, MA) to create a new plasmid, pAC10. The predicted translation product from the *hlh-8* locus of pAC10 will have the first 9 amino acids of CeTwist plus 6 nonsense amino acids and a premature stop codon but will leave any translation product from the upstream ORF intact. pAC10 was injected into *hlh-8 (nr2061)* animals at a concentration of 25 μg/ml.

Reporter strain constructions

We used standard genetic methods to introduce a set of characterized *gfp* fusion transgenes into an *hlh-8 (nr2061)* genetic background. Animals that were homozygous for both the *gfp* transgene and *hlh-8 (nr2061)* were analyzed. *gfp* reporter strains used were the following: *egl-15::gfp ayIs2(IV)* (Harfe et al., 1998a), *NdEbox::gfp ccIs4656(IV)* (Harfe and Fire, 1998), *hlh-8::gfp ayIs7(IV)* (Harfe et al., 1998b), *myo-3::secreted gfp arIs39(X)* (J. Fares and I. Greenwald, personal communication), *pal-1::gfp ctIs33* (L. Edgar and W. B. Wood, personal communication) and *myo-3::gfp ccIs4251(I)* (Fire et al., 1998). We confirmed that the *egl-15::gfp* and *NdEbox::gfp* reporters were present and homozygous, but not expressed, in *nr2061* strains by outcrossing the strains to wild-type (N2) males and observing that all of the cross progeny expressed *gfp*.

Immunofluorescence

To observe staining of the anal depressor and anal sphincter, we freeze/cracked worms in water and fixed them by incubating in methanol followed by acetone. Rhodamine phalloidin (Molecular Probes; Eugene, Oregon) (0.03 U/μl in PBS) was added to the fixed animals that were examined without washing. To visualize the intestinal muscles, we used the methanol/acetone fixation, as above, and used a 1:40 dilution in TTBS (100 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween 20) of the monoclonal antibody 3NB12 (Okamoto and Thomson, 1985). This antibody was previously shown to stain enteric muscles and a subset of pharyngeal muscles (Priess and Thomson, 1987). The slides were incubated overnight at 4°C, and after washing with TTBS the slides were incubated in a 1:400 dilution in TTBS of rhodamine-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA). Photographs of the anal region and of *gfp* markers were all taken with a SenSys camera (Photometrics, Tuscon, Arizona); imaging was performed using IPLabs software (Scanalytics, Fairfax, VA).

RESULTS

A deletion in the *hlh-8* coding region

In our effort to understand the contributions of bHLH transcription factors to *C. elegans* muscle development, we sought to evaluate the loss-of-function phenotype for the *C. elegans* Twist gene, *hlh-8*. An allele of *hlh-8, nr2061,* had been obtained in a PCR-based screen of a deletion library by L. Liu and colleagues (1999). The exon-intron boundaries for the *hlh-8* locus were previously determined by sequencing cDNA (Fig. 1; Harfe et al., 1998b). We sequenced the *hlh-8* exons and splice boundaries in the homozygous *nr2061* animals and found the genomic DNA had a deletion of 1267 base pairs spanning base pairs 1148-2415 (with the A in the initiator codon ATG numbered 1). The deleted locus was missing approximately half of the first intron, both exon 2 and intron 2, and almost 90% of exon 3 (Fig. 1). The wild-type CeTwist protein is 178 amino acids in length including 57 amino acids in the bHLH domain. The predicted translation product that might be expressed from the *hlh-8 (nr2061)* locus would be 49 amino acids. This protein would include the basic domain plus

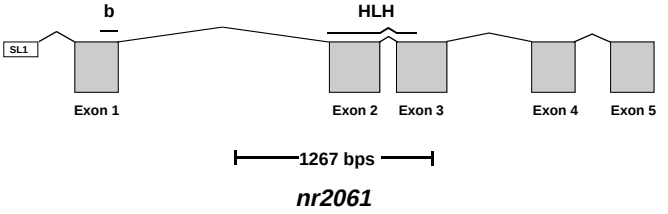

Fig. 1. *hlh-8 (nr2061)* animals lack a CeTwist HLH domain. A diagram of the five exons of the *hlh-8* genomic sequence. Reverse transcriptase reactions followed by PCR were used to examine the beginning of the *hlh-8* mRNA; SL-1 was found to be *trans*-spliced to the 5′ end of the *hlh-8* message (Krause and Hirsh, 1987). As indicated above the gene, the bHLH region spans three exons. Using PCR and standard DNA sequencing, the *nr2061* allele was found to have a deletion in *hlh-8* that removes the HLH domain in the CeTwist protein.

2 amino acids of the HLH domain from exon 1, followed by 15 nonsense amino acids and a premature stop codon. Because the deletion in the *hlh-8* locus of *nr2061* animals removed virtually the entire HLH domain, it would be expected that any protein products would be unable to dimerize and thus unable to bind DNA. This predicted lack of activity of CeTwist in *nr2061* animals made it reasonable to believe that these animals were null for *hlh-8* and their mutant phenotype reflected a lack of CeTwist function in the worms. Additionally, this allele was completely recessive: *hlh-8 (nr2061)*/+ animals did not display any detectable mutant phenotypes. Additional evidence for the null character of *hlh-8 (nr2061)* came from the lack of activity of several characterized *hlh-8* target genes in these animals (see later section). *hlh-8* did not appear to have an obvious maternal contribution. Homozygous progeny born from heterozygous animals were indistinguishable from progeny born from homozygous animals.

Functional striated muscles in *hlh-8 (nr2061)* animals

The first striking property of *hlh-8 (nr2061)* animals was that they were motile and grew to produce fertile adults. *C. elegans* moves by using a set of longitudinally contracting striated muscle bands. These muscles are formed predominantly during embryogenesis (81 cells) although 14 cells are added during larval development. The wild-type motility of the *nr2061* animals indicated that these muscle bands had formed and were functional. Motility is not a direct proof that all embryonic muscles are present. Schierenberg and Junkersdorf (1992) found that ablating an embryonic precursor cell responsible for the formation of 20 out of the 81 embryonically derived muscle cells resulted in motile animals with substantially decreased muscle numbers. To be certain that the proper number of muscles were being formed during embryogenesis of *hlh-8 (nr2061)* animals, we counted body wall muscles in newly hatched L1 larvae using a *myo-3::gfp* reporter (Table 1). We found no significant difference in larval body wall muscle number between wild-type and *hlh-8 (nr2061)* animals or a constipated control strain, *aex-1 (sa9)*.

Loss of function in *hlh-8* caused egg-laying and defecation phenotypes

Because CeTwist is found in a restricted number of cells, including the enteric muscles and the M mesoblast and its descendants that give rise to sex muscles (Fig. 2), we predicted that animals with a mutation in the *hlh-8* gene might have defects in sex muscle and/or enteric muscle function. Indeed, the *hlh-8 (nr2061)* animals had both phenotypes. The mutant animals were 100% egg-laying defective (Egl). All embryos were retained inside the hermaphrodites and continued developing until they hatched to cause the ‘bag of worms’ phenotype (Greenwald, 1997). The *hlh-8* mutant animals also had an obvious clear region from the base of the pharynx in the anterior of the animal to the anus in the posterior due to an expanded intestinal lumen which is a hallmark of a constipated (Con) phenotype. In contrast, it is difficult to discern the intestinal lumen of wild-type worms using the dissecting microscope. A third visible phenotype in these mutant worms was in the tail region. In comparison to the smooth and tapered tail of wild-type hermaphrodites, *hlh-8 (nr2061)* animals had a wider diameter (a bump approximately 1.5 times larger than the diameter of wild-type) near their anus. The mutant animals were also smaller and grew slower than wild-type worms at all larval and adult stages. Other severely constipated mutants frequently have these growth defects due to malnourishment (Avery and Thomas, 1997).

To determine if the deletion in *hlh-8* was the primary cause of the phenotypes in the mutant animals, we attempted to rescue the defects with wild-type *hlh-8* genomic DNA. We

Table 1. Body wall muscle counts in L1 larvae

Number of larval body wall muscles (*myo-3::gfp*)*	Wild type	*aex-1 (sa9)*	*hlh-8 (nr2061)*
78	0%	0%	5%
79	0%	0%	0%
80	12%	6%	15%
81	88%	94%	80%
n	16	17	20

*Method used to evaluate the phenotype. A lower count than expected was observed in some wild-type and mutant animals. This observation may reflect a low level of mosaicism in the *myo-3::gfp* transgene.

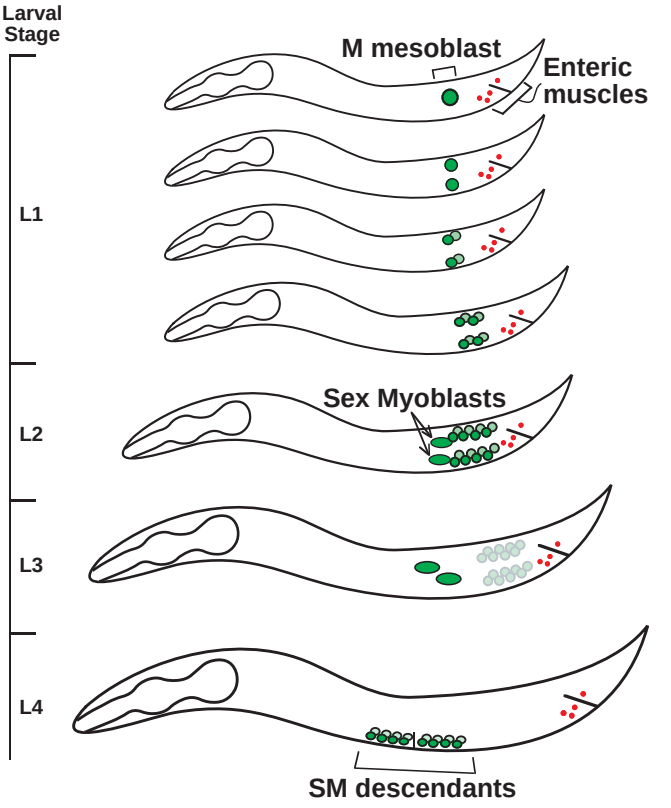

Fig. 2. Larval distribution of *hlh-8* gene products. This line drawing shows the *C. elegans* larval stages (not to scale) and the cells where CeTwist is found based on antibody staining and a *gfp* reporter (data from Harfe et al., 1998b). The *hlh-8::gfp* reporter is also expressed in several putative head neurons (not shown). The green circles represent the nuclei where *hlh-8::gfp* expression is seen in the M mesoblast and descendants (for lineage information, see Fig. 4). The red circles represent the nuclei of the enteric muscles that are observed with indirect immunofluorescence using a CeTwist antibody.

found that all three of the phenotypes (Egl, Con, and tail bump) could be rescued in transgenic *hlh-8 (nr2061)* animals containing the genomic *hlh-8* open reading frame plus 9 kb upstream and 3 kb downstream of genomic DNA (pBH64). Greater than 90% of animals that have the transgene (as marked by the dominant *rol-6* marker) could lay eggs and at least 50% of these animals were no longer Con and had a wild-type looking tail (n>34 for each of two lines). We could also obtain full or partial rescue of these phenotypes in greater than 50% of mutant animals that expressed *hlh-8* cDNA plus 500 base pairs upstream of the start of transcription (n>46 for each of two lines). As a negative control, a plasmid with a frameshift mutation (pAC10) failed to rescue *hlh-8 (nr2061)* transgenic animals (n>30 for each of two lines). The rescue phenotype together with the molecular lesion found in the *hlh-8* locus of the mutant animals led us to conclude that the absence of CeTwist function was responsible for the phenotypes that we observed in the *nr2061* allele.

hlh-8 mutant animals failed to develop wild-type defecation muscles

We sought to determine why the *hlh-8 (nr2061)* animals were constipated. We used rhodamine phalloidin to observe the dorsoventrally oriented actin filaments of the anal depressor and the annular sphincter in wild-type and mutant animals (shown diagrammatically in Fig. 3A and the data in Fig. 3C-J). In *hlh-8 (nr2061)* animals, the anal depressor staining was either absent or was observed as wispy remnants (Fig. 3G,H). Wild-type staining of the depressor muscle reappeared in transgenic *nr2061* animals that harbored a wild-type genomic *hlh-8* plasmid (Fig. 3I,J). To ensure that constipation did not cause anal depressor degeneration, we also stained constipated *aex-1 (sa9)* animals that had defects in body wall contractions necessary for defecation (Thomas, 1990). Wild-type anal depressors were observed in *aex-1 (sa9)* animals. Taken together, these data demonstrate the absence of a differentiated depressor muscle in *hlh-8 (nr2061)* animals.

A second set of enteric muscles are the flat intestinal muscles that are closely associated with the posterior gut (Fig. 3A; Sulston et al., 1983). To observe the intestinal muscles in our mutant, we used the tissue-specific monoclonal antibody 3NB12 (Okamoto and Thomson, 1985). We observed staining of the large cell body and distinctive finger-like projections of the intestinal muscles in wild-type and *aex-1 (sa9)* animals (Fig. 3K-N) but could not see these muscles in the *hlh-8 (nr2061)* animals above the background staining of the body wall muscles (Fig. 3O,P). The intestinal muscles were present in the rescued *hlh-8* mutant animals expressing wild-type *hlh-8* (Fig. 3Q,R).

In contrast, we saw little or no effect of the *nr2061* allele on a third type of enteric muscle, the anal sphincter muscle. In a single plane of focus, we could observe two dots of staining resulting from sectioning of the annular muscle in two places (Fig. 3A). Staining of the anal sphincter was observed in wild-type *C. elegans* as well as in the *nr2061* animals (Fig. 3C-J). The anal sphincter in *hlh-8 (nr2061)* animals appeared to have a wider diameter than the sphincter of wild-type worms, possibly due to stretching from the expanded intestinal

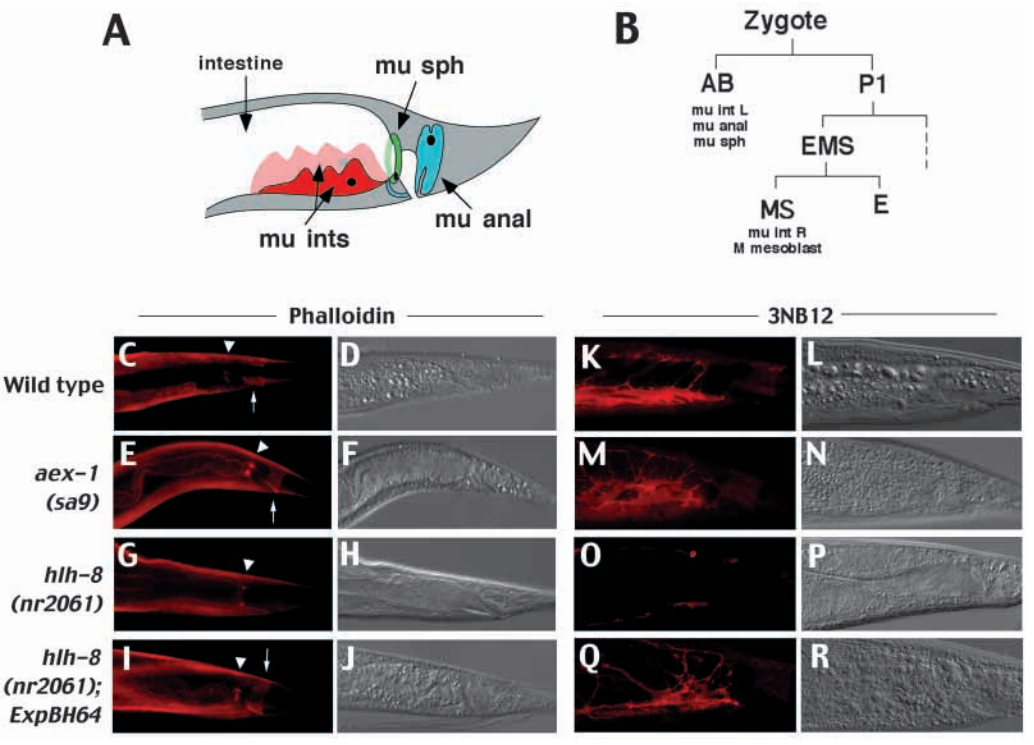

Fig. 3. The *hlh-8 (nr2061)* mutant has defects in some enteric muscles. (A) A diagram of the worm tail region showing the location of the enteric muscles. mu sph, anal sphincter; mu anal, anal depressor; mu ints, intestinal muscles (adapted from White et al., 1986). (B) A partial lineage to illustrate that the enteric muscles come from two separate founder cells (adapted from Sulston et al., 1983). (C-J) Immunofluorescence experiments on young adult hermaphrodites stained with rhodamine phalloidin (C,E,G,I) with Nomarski images (D,F,H,J). All of the animals shown have anal sphincters that appear as two dots in these optical cross sections (white arrowheads). *hlh-8 (nr2061)* animals do not have anal depressors, whereas wild-type (N2), *aex-1 (sa9)*, and *nr2061* animals expressing *hlh-8* from an extrachromosomal array do have depressors (white arrows). (K-R) Immunofluorescence experiments of older adult hermaphrodites stained with 3NB12 monoclonal antibody (K,M,O,Q) with Nomarski images (L,N,P,R). The bright staining on the ventral side of the worms is the mu int of the left side (the right mu int is out of the focal plane). *hlh-8 (nr2061)* animals lack patterns of 3NB12 staining that resemble wild-type intestinal muscles. (A,C-R) All images are oriented with posterior to the right and dorsal to the top. Staining along the length of the animals is present in all strains and identifies body wall muscles.

diameter in the mutants although this is not so apparent in the constipated *aex-1 (sa9)* strain. In an independent set of experiments, we used the myosin heavy chain-specific monoclonal antibody 5-6 (Miller et al., 1983) to observe the enteric muscles. The myosin staining also revealed apparently normal anal sphincters in the *nr2061* animals (data not shown).

In Fig. 3B, we have reproduced part of the *C. elegans* lineage (Sulston et al., 1983) highlighting the distinct founders from which the enteric muscles are derived. Three out of four of the enteric muscles, from two separate lineages, were not formed properly in *hlh-8 (nr2061)* animals. This muscle loss caused the constipated phenotype in these animals and may also be the cause of the tail bump phenotype due to a loss of structural integrity in the anal region. It is interesting to note that the cells affected in the *hlh-8 (nr2061)* animals are derived from different embryonic founder cells (AB and P1). These results indicated that lineage-specific restriction of *hlh-8* activity is unlikely.

As a first step to characterize the enteric muscle malformation defect in *hlh-8 (nr2061)* development, we asked whether the four enteric muscle cells were born in the *nr2061* embryo. We considered the lineage of CeTwist-expressing cells in wild-type animals (Fig. 3B; Sulston et al., 1983). The left intestinal muscle (mu int L) and the anal depressor (mu anal) are sister cells; the right intestinal muscle (mu int R) has a sister cell that undergoes programmed cell death; the anal sphincter (mu sph) and the sole AB-derived body wall muscle are sister cells; and the M mesoblast (discussed more in the next section) has a sister cell that undergoes programmed cell death. To visualize these cells in the embryo, we used a *pal-1::gfp* reporter. This reporter is expressed in the 4 enteric muscles, the AB-derived body wall muscle, and the M mesoblast; additional expression is seen in several hyps and the tail spikes in the posterior of wild-type 1 1/2 fold embryos (L. Edgar and W. B. Wood, personal communication). We expected that any major differences between wild-type embryos and *nr2061* embryos should be revealed by this marker. For example, if the precursor to mu int L and mu anal does not divide, we would expect to find one fewer cell in the mutant animals. We crossed the integrated *pal-1::gfp* marker into *hlh-8 (nr2061)* worms and compared the number of *gfp*-expressing cells in the tail region of these embryos in comparison to wild-type *pal-1::gfp* embryos. We counted 13.7±0.8 cells in the tail region of wild-type worms (n=32) and 13.9±0.4 *pal-1::gfp* positive cells in the *hlh-8 (nr2061)* worms (n=22). Because of the identical numbers of *pal-1::gfp* expressing cells in the two strains, we concluded that presumptive enteric muscles were at least born and that differentiation defects must have occurred later in development. This conclusion was further supported by our observations of apparent wispy remnants of the affected muscles in some *nr2061* animals.

hlh-8 mutant animals had defects in M lineage patterning

At hatching, wild-type worms have a single non-gonadal mesodermal blast cell, M, that divides characteristically to give rise to most of the post-embryonic mesodermal cells (Sulston

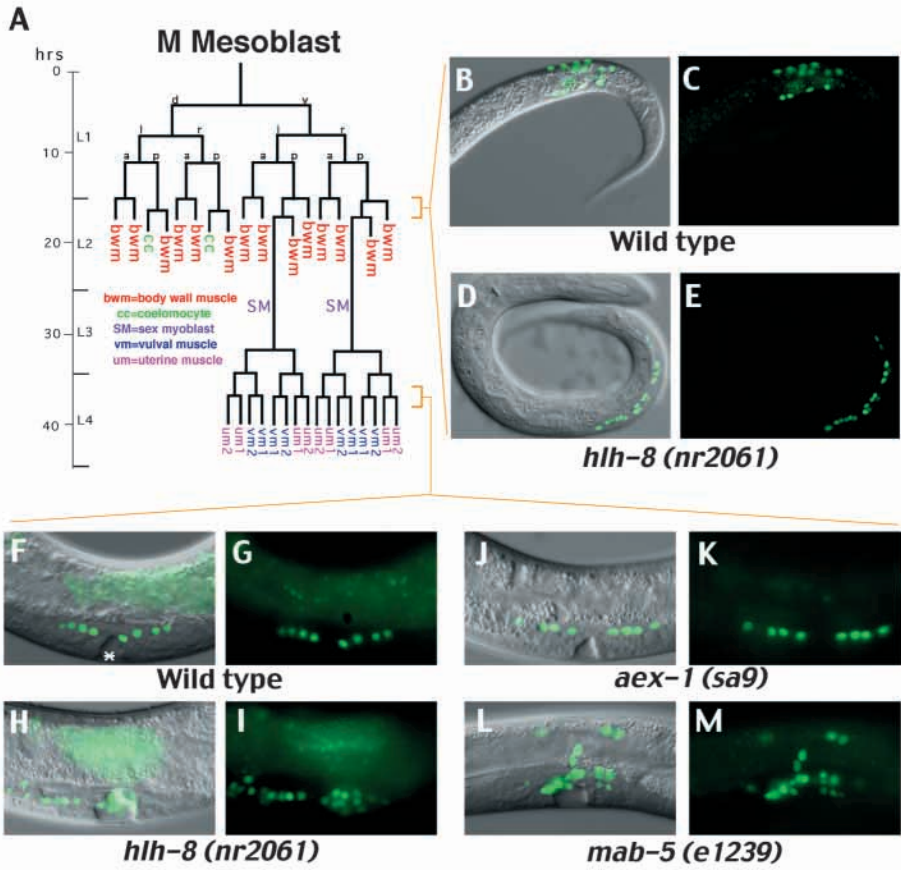

Fig. 4. *hlh-8 (nr2061)* animals have defects in M lineage patterning. (A) The lineage from the M mesoblast, including the differentiated mesodermal cell types (adapted from Sulston and Horvitz, 1977). On the left the four larval stages are indicated. On the right side of the lineage as indicated by brackets are the stages of the animals shown in the rest of the figure. (B-M) All worms shown contain integrated *hlh-8::gfp ayIs7(IV)*, are oriented with posterior to the right and ventral to the bottom, and are shown as either merged Nomarski/GFP images or as GFP only images. (B,C) Wild-type (N2) and (D,E) *hlh-8 (nr2061)* larvae at the beginning of L2 stage are shown. The wild-type worm has 16 M great-great granddaughters with one quadrant of cells out of the plane of focus. The *nr2061* larva has 18 M great-great granddaughters (2 of which are presumably the SMs) all in a single focal plane on the ventral side of the animal. (F-M) The region of the developing vulva of L4 larvae are shown. The vulval opening (white asterisk) is indicated in F and is in the same orientation in all animals shown. The Nomarski and GFP images are taken in two different focal planes to see the developing vulva and at least half of the SM descendants concurrently. (F,G) Wild-type and (J,K) *aex-1 (sa9)* animals have 16 SM descendants with only half visible in this focal plane. (H,I) An example of an *hlh-8 (nr2061)* animal with approximately 22 SM descendants. (L,M) An example of a *mab-5 (e1239)* animal with approximately 25 SM descendants.

and Horvitz, 1977). Differentiated M lineage mesodermal cells include 2 coelomocytes, 14 striated body wall muscles and 16 non-striated sex muscles (vulval and uterine) as shown in the lineage in Fig. 4A. Because the *hlh-8* promoter was previously shown to be active in M and its descendants (Harfe et al., 1998b) and because *hlh-8 (nr2061)* mutants were Egl and possibly had defective sex muscles, we used a variety of means to follow M divisions and determine whether M cell descendants were formed in the mutant animals. The first tool for this analysis was an *hlh-8::gfp* reporter (Figs 2, 4; Table 2).

The *hlh-8::gfp* reporter construct is expressed in M and its descendants prior to differentiation (Fig. 2; Harfe et al., 1998b). This reporter does not contain full length *hlh-8* and did not alleviate the phenotypes of *nr2061* animals. The first observation we made in the *hlh-8 (nr2061); hlh-8::gfp* animals was that the *gfp* was expressed in all L1 larvae in a single large cell resembling the wild-type M mesoblast in the posterior region of the worms. Because the *hlh-8::gfp* does not provide *hlh-8* activity, this GFP pattern suggested that the *hlh-8* gene expression in M is not dependent on positive autoregulation. We next followed the divisions of M in several worms using GFP fluorescence. The first division of M in wild-type animals occurs dorsoventrally followed by left-right divisions resulting in 4 quadrants of M granddaughters. These cells then proceed to divide twice anterioposteriorally producing 4 quadrants of 4 cells each (M great-great granddaughters) (Figs 2, 4A-C). We found that division planes were randomized in the first two divisions of *nr2061* animals when compared with wild-type *hlh-8::gfp* worms. In *hlh-8 (nr2061)* animals, the first division often results in two M daughters on the ventral side of the animals (Table 2) with the subsequent divisions occurring exclusively anterioposteriorally resulting in a single, ventral queue of M great-great granddaughters (Fig. 4D,E). We used *aex-1 (sa9); hlh-8::gfp* as a control for secondary effects of constipation and did not observe any significant deviations from the wild-type M lineage divisions (Table 2).

At the beginning of the L2 larval stage, the two M-derived sex myoblasts (SMs) of the hermaphrodite are born (Fig. 4A; Table 2). The SMs migrate to the central gonad area and divide to become the 16 non-striated sex muscles (vulval and uterine muscles) (Sulston and Horvitz, 1977). Prior to sex muscle differentiation, wild-type L4 larvae (and constipated *aex-1 (sa9)* larvae) invariably have 16 *hlh-8::gfp*-expressing SM descendants in 2 focal planes flanking the developing vulva (Figs 2; 4F,G,J,K). We found that *hlh-8 (nr2061)* animals had a variable number of SM-like cells ranging from 1 to 6 in total (Table 2) and later showed a variable number of extra SM descendants. 26 out of 26 *hlh-8 (nr2061)* L4 larvae we surveyed had *hlh-8::gfp*-positive wild-type-like SM descendants in the central region of the worm near the vulva (Fig. 4H,I; Table 2). SM-like cells could also be observed in the posterior of many *hlh-8 (nr2061)* animals, possibly reflecting incompletely migrated SMs. In addition to the variable number of SMs in *hlh-8 (nr2061)* animals, we noticed that the *hlh-8::gfp* persisted into adulthood in some worms. In wild-type animals, the last time that *hlh-8::gfp* can be seen is in precursors to sex muscles when the expression fades away and is not seen again until the next generation (Harfe et al., 1998b).

Kenyon (1986) has observed extra and incompletely migrated SMs in *mab-5* (HOM-C) mutants. We assayed SM descendants in *mab-5 (e1239)* animals using *hlh-8::gfp* and observed extra SM descendants in the gonad region (Fig. 4L,M). This phenotype was strikingly similar to the phenotype we observed in *hlh-8 (nr2061)* animals.

Table 2. M lineage descendants in a CeTwist mutant

Early M division planes*,‡	Wild type	*aex-1 (sa9)*	*hlh-8 (nr2061)*
D/V	100%	100%	21%
V	0%	0%	75%
D	0%	0%	4%
n	19	51	28
Number of sex myoblast-like cells per animal‡			
1	0%	0%	5%
2	100%	100%	38%
3	0%	0%	26%
4	0%	0%	19%
5	0%	0%	7%
6	0%	0%	5%
n	21	13	42
Divided SMs‡			
Yes	100%	100%	100%
No	0%	0%	0%
n	15	25	26

*After the first division of M, Wild Type (N2) animals have one dorsal (D) and one ventral (V) M daughter. D/V = 1D and 1V cell; V = 2 V cells; D = 2 D cells.

‡Observations made with animals that were expressing integrated *hlh-8::gfp*.

hlh-8 (nr2061) animals had variable defects in differentiated M descendants

Given defects in the early M divisions and in SM number (Table 2), we were interested in knowing whether the differentiated M descendants (vulval muscles, coelomocytes, and body wall muscles) were properly formed in the *hlh-8* mutant animals. We observed the mutants with polarized light to visualize the filaments of the vulval muscles. Although the vulval muscles were readily visible in wild-type and *aex-1 (sa9)* animals, normal vulval muscles were not evident surrounding the *hlh-8 (nr2061)* vulva (Table 3). Nonetheless, some of the muscle differentiation program is active in the SM lineages. In wild-type worms, the 'X' configuration of the vulval muscles attached to the vulval opening can be seen with the *myo-3::gfp* marker (Fig. 5A,B). The SM descendants in *hlh-8 (nr2061)* animals were capable of activating a *myo-3::gfp* fusion; this activation was observed as small cells in the vulval region of *hlh-8 (nr2061)* animals (Fig. 5D,F). In many *hlh-8 (nr2061)* animals a subset of small *myo-3::gfp* positive cells appeared to partially attach to the vulval opening (Fig. 5C,D). These cells also exhibited birefringence. However, none of the animals had wild-type vulval muscles. The lack of well-formed vulval muscles that could be visualized by their birefringence (Table 3) indicated that only partial differentiation of these SM-derived cells has occurred. To rule out the possibility that the partially formed vulva muscles failed to function due to improper innervation, we

have also tested the ability of *hlh-8 (nr2061)* animals to lay eggs in response to exogenous serotonin or imipramine (drugs that stimulate wild-type animals to lay eggs). A lack of response to either drug is interpreted as a defective vulva and/or sex muscles rather than neuronal defects (Trent et al., 1983). In controlled experiments, where wild-type animals responded to the drugs (n=30), *hlh-8 (nr2061)* animals did not lay eggs in response to either serotonin (n=30) or imipramine (n=30). Taken together, these data suggested that *hlh-8 (nr2061)* animals were unable to lay eggs because they did not have functional vulval muscles.

Several body wall muscles are also derived from the M mesoblast (see Fig. 4A). To observe the number of M-derived body wall muscles that differentiated in *hlh-8 (nr2061)* animals, we counted the total number of body wall muscles in adult animals. Both wild-type animals and the constipated *aex-1 (sa9)* animals had an invariant number (95±0) of body wall muscles (n>14; Table 3). Because the *hlh-8* mutant animals had extra SM-derived cells expressing the *myo-3::gfp* reporter in the vulval region (Fig. 5E,F) and occasionally in the posterior, we were careful to distinguish the larger body wall muscle nuclei from the smaller SM descendant nuclei in these animals (we disregarded any worm where the distinction was ambiguous). We found that *hlh-8* mutant animals had a variable number (91.9±3.7) of body wall muscles (n=21). Out of the 21 mutant worms that we could count with confidence, we saw only 2 worms with a wild-type number of muscles (Table 3). We observed 15 worms that were missing one or more body wall muscles with the lowest number being 86 and 4 worms with extra body muscles (3 with just 1 extra and 1 with 5 extra muscles; Table 3).

To observe the formation of another differentiated M descendant, we used the *myo-3::secreted gfp* reporter, which is taken up by coelomocytes (Harfe et al., 1998a), in combination with Nomarski optics. Whereas the majority of *hlh-8 (nr2061)* animals had the normal number of coelomocytes, approximately one fourth of the mutant animals that we observed had fewer than the wild-type number of coelomocytes (Table 3). The variability that we saw with extra M-derived cells (sex myoblasts or body wall muscles) or with missing M-derived cells (sex myoblasts, coelomocytes, or body wall muscles) suggests that there is not an explicit and penetrant cell-specific transformation phenotype in *hlh-8 (nr2061)* worms but that CeTwist contributes to the proper patterning of differentiation in this mesodermal lineage.

Table 3. Differentiated M descendants in a CeTwist mutant

Normal vulval muscles (polarized light)*	Wild type	*aex-1 (sa9)*	*hlh-8 (nr2061)*
Yes	100%	100%	0%‡
No	0%	0%	100%
n	21	22	29
Number of coelomocytes per animal (*myo-3::secreted gfp*)*			
4	0%	0%	14%
5	0%	0%	10%
6	100%	100%	76%
n	16	38	29
Number of adult body wall muscles (*myo-3::gfp*)*,§			
86-94	0%	0%	80%
95	100%	100%	5%
96-100	0%	0%	15%
n	25	14	40

*Method used to evaluate the phenotype.

‡Under higher resolution analysis, 30% of animals (n=23) had 1-3 birefringent muscle remnants at the vulva that were *myo-3::gfp* positive but failed to fully differentiate as vulval muscles.

§These muscle counts also involved Nomarski inspection of muscle quadrants. This method provides more precise counts than are possible in the small L1 animals reported in Table 1.

Two CeTwist targets were silent in *hlh-8 (nr2061)* mutant animals

Several potential CeTwist targets have been identified, including the NK-2 homeodomain protein, *ceh-24*, and the fibroblast growth factor (FGF) receptor homolog, *egl-15* (Harfe et al., 1998b). *hlh-8* misexpression results in concordant misexpression of *gfp* reporters derived from these genes (Harfe et al., 1998b). Reciprocally, a decrease in the *gfp* reporter expression was seen when RNA-mediated interference using *hlh-8* cDNA was performed (Harfe et al., 1998b). In order to assess the nature of *hlh-8 (nr2061)* and to determine if CeTwist is necessary for expression of these reporters, we crossed each into the *hlh-8 (nr2061)* background. In wild-type animals, *egl-15::gfp* is expressed in the four vm1 (vulval) muscles and the *NdEbox::gfp* reporter containing a concatomerized enhancer element located upstream of *ceh-24* is expressed in the vm1, vm2, uterine, and intestinal muscles as well as the anal depressor (Fig. 6A,B; Harfe and Fire, 1998; Harfe et al., 1998b). None of these non-striated muscles had any *gfp* expression in *hlh-8 (nr2061)* animals containing either reporter (Fig. 6C,D; and not shown). Reporter expression for *egl-15::gfp* and *NdEbox::gfp* was restored in transgenic *nr2061* animals expressing the wild-type *hlh-8* gene (Fig. 6E,F and data not shown). This data indicates that CeTwist is necessary for activating expression from *egl-15* and *ceh-24* promoters.

DISCUSSION

We have characterized a deletion allele of the gene that codes for CeTwist (*hlh-8*). Twist homologs in other organisms have been shown to be involved in mesoderm development, and we have found that CeTwist also plays a role in the *C. elegans* mesoderm. Although embryonic development of striated muscle was normal, the lack of functional CeTwist caused a subset of embryonic non-striated and postembryonic mesodermal cells to develop improperly. As a result, these mutants were missing functional non-striated sex muscles and a subset of enteric muscles. Affected cells included 3 out of the 90 embryonically born mesodermal cells and a majority of the post-embryonic mesoderm. The severity of the defects in the intestinal muscles and the anal depressor suggested that CeTwist is essential for their differentiation. On the other hand, the ability of a subset of the post-embryonic M-derived cell types to form suggested that CeTwist contributes to, but is not

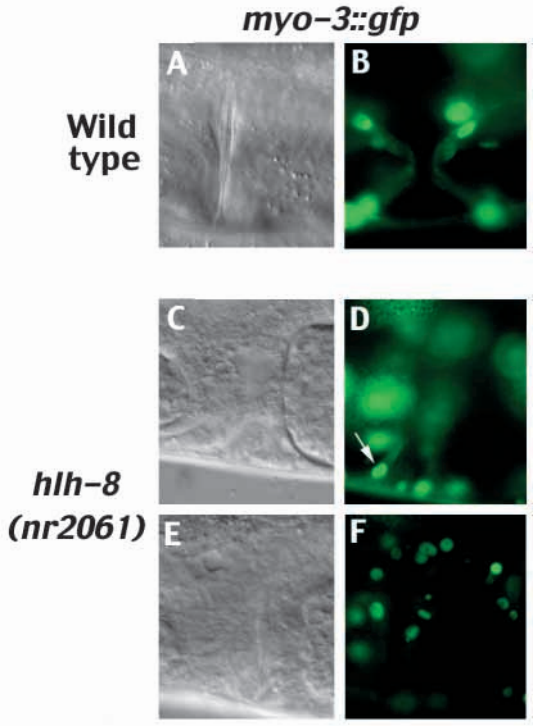

Fig. 5. *hlh-8 (nr2061)* animals appear to make vulval-like muscle connections. (A-F) Ventral view of the vulval opening of animals containing *myo-3::gfp ccIs4251(I)*. (A,C,E) Nomarski and (B,D,F) GFP images are shown. (A,B) Wild-type (N2) adults have 8 vulval muscles (4 seen in this focal plane) that attach to the epithelium surrounding the vulval opening (in an 'X' arrangement). Nuclei are intensely green and fainter GFP is seen in the cytoplasm. (C-F) An example of an *hlh-8 (nr2061)* adult with approximately 19 SM descendant-like cells (with smaller nuclei than the surrounding larger body wall muscle nuclei). In the first focal plane (C,D), a vulval-like muscle making a connection near the vulval opening is marked by a white arrow. Faint GFP is visible in the cell body projecting upward from the intensely fluorescing nucleus. This animal also has a second muscle-like cell that is out of focus. In the second focal plane (E,F), at least 10 of the SM descendant-like cells can be seen. These cells do not appear to be making any muscle-like projections.

absolutely required for, the development of these cells. Because we saw muscle-like cells in the vulval region of *hlh-8 (nr2061)* animals, we expect that CeTwist is not required for myoblast fate but for a pattern of proper differentiation.

The primary defects that we observed in *hlh-8 (nr2061)* animals were relatively late developmental events. The cells that become the enteric muscles were born; however, intestinal muscles and the anal depressor were not formed correctly in the mutant animals. Although no cells in the posterior of the *hlh-8* mutant animals expressed the *ceh-24* reporter that is normally expressed in the intestinal muscles and the anal depressor, we occasionally saw apparent remnants of these muscles, suggesting that these cells were myoblast-like but unable to execute the correct developmental program. Similarly, many *hlh-8 (nr2061)* animals progressed quite far in development of the M lineage. SM descendant-like cells proliferated near the vulva and activated *myo-3::gfp*. Moreover, the cells occasionally appeared to make muscle-like attachments to the vulva, although functional sex muscles were never formed and 100% of the animals retained their eggs.

The phenotypes that we observed in *hlh-8* mutant worms have parallels with null mutations in mice and flies. In *M. musculus*, *twist* null homozygotes are embryonic lethal, but these animals survive until embryonic day 11 and have detectable mesodermal development with tissue-specific defects (Chen and Behringer, 1995). A complicating issue is that a number of additional mouse *twist* homologs and related bHLH factors have been identified that may make contributions to other aspects of early mesoderm patterning (Blanar et al., 1995; Burgess et al., 1995; Cserjesi et al., 1995; Li et al., 1995). To date, the *C. elegans* genome is approx. 99% complete and no other worm *twist* homolog has been identified (Ruvkin and Hobert, 1998). *D. melanogaster twist* null mutants die as embryos that completely lack any mesoderm (Simpson, 1983; Nüsslein-Volhard et al., 1984). The severity of the fly mutants may reflect an early developmental requirement for Twist in

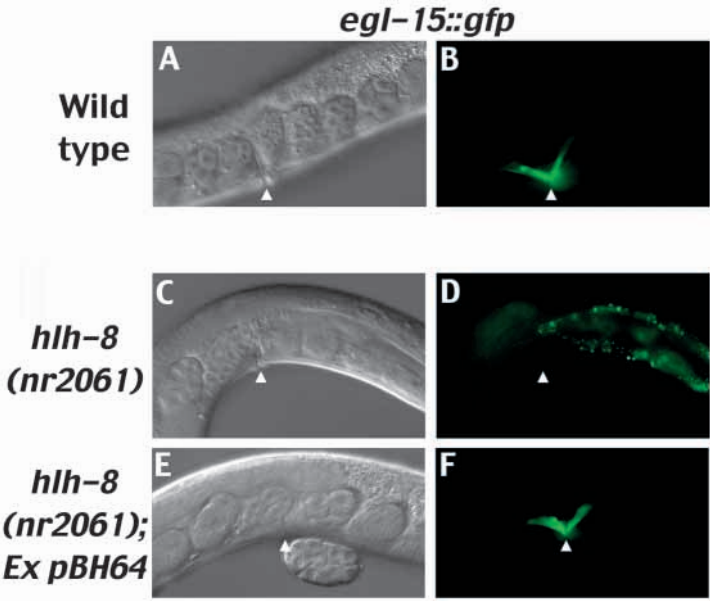

Fig. 6. Effect of hlh-*8 (nr2061)* on target gene reporters. *egl-15*, encoding an FGF receptor homolog, and *ceh-24*, encoding an NK-2 class homeodomain homolog, have been shown by Harfe et al., (1998b) to be targets of CeTwist. (A,C,E) Nomarski and (B,D,F) GFP images; lateral views. Animals are oriented with dorsal to the top and posterior to the right. The vulval opening in the adult hermaphrodites is marked with a white arrowhead. (A,B) The wild-type vulval (vm1) expression of the *egl-15::gfp* is seen in two vulval muscles with the other two in a different focal plane. (C,D) *egl-15::gfp* is not expressed in *hlh-8 (nr2061)* animals. The green dots present in this worm are from autofluorescing gut granules. (E,F) Wild-type *egl-15::gfp* expression is restored in *hlh-8 (nr2061)* animals rescued with a plasmid containing *hlh-8* genomic DNA (pBH64). An enhancer element from *ceh-24* (*NdEbox::gfp*) is expressed in all of the sex muscles, in the anal depressor, and in the intestinal muscles of wild-type worms. This reporter is also not expressed in *hlh-8 (nr2061)* animals and reappears in rescued animals (not shown).

mesoderm specification that is not found in mice and worms. If Twist is supplied to the early fly embryo, the defects that are seen later in development are restricted to subsets of muscle cells such as executing the choice between somatic and visceral mesoderm (Baylies and Bate, 1996) and controlling mesodermal patterning (Cripps and Olsen, 1998). Taken together, the data from mice, flies and worms suggest that a critical subset of *twist* functions may be conserved evolutionarily.

During our investigation of the defecation defects in the *hlh-8 (nr2061)* animals, we noticed an interesting enteric muscle pattern. Of the four worm enteric muscles, only the anal sphincter appeared to be intact, by immunostaining. This result suggests that the sphincter muscle, sister of a body wall muscle, has a unique developmental mechanism among these four enteric muscle cells. Another worm mutant, *lin-12(q269) glp-1(q231),* has a similar phenotype: the intestinal muscle and anal depressor are absent but the sphincter muscle is intact (Lambie and Kimble, 1991). Also, the phenotype of the tail bump in the posterior of *hlh-8 (nr2061)* animals is reminiscent of the *lag-1 (q416)* (for **l**in-12 **a**nd **g**lp-1, Suppressor of hairless homolog) mutant tails (Lambie and Kimble, 1991). Both *lin-12* and *glp-1* are homologs of the *D. melanogaster* cell-cell signaling molecule Notch (Yochem et al., 1988; Yochem and Greenwald, 1989), and both genes function through *lag-1* (Christensen et al., 1996). The similar phenotypes of *hlh-8 (nr2061)* and *C. elegans* Notch pathway mutants may indicate that CeTwist is interacting in the enteric muscles with signaling through the Notch pathway. Further circumstantial evidence for Notch pathway interactions with CeTwist is that *lin-12* functions later in the M lineage where we detected defects in *hlh-8 (nr2061)* animals. In the M lineage, the main cellular transformations of *lin-12* are sex myoblasts to coelomocytes (SM→cc) in loss-of-function (0) mutants and coelomocytes to sex myoblasts (cc→SM) in dominant gain of function (d) mutants (Greenwald et al., 1983; Table 4). We have observed a variable number of extra SMs in *hlh-8 (nr2061)* animals and fewer coelomocytes. It is possible that the cc→SM transformations in the *hlh-8 (nr2061)* reflect novel *lin-12*-mediated interactions among the mispositioned M descendants. In wild-type animals, *hlh-8* expression occurs earlier than *lin-12* expression in the M lineage (Wilkinson and Greenwald, 1995). *hlh-8* could therefore act upstream of *lin-12* by ensuring that only certain M descendants are competent for *lin-12*-mediated cell-cell interactions. In *Drosophila* adult indirect flight muscle development, Twist regulation involves Notch signaling (Anant et al., 1998), and a similar situation might apply to *C. elegans*.

The HOM-C homolog, MAB-5, has been proposed to control at least some aspects of *hlh-8* expression because *hlh-8::gfp* is off early in the M lineage in *mab-5 (e1239)* mutants and comes on later when *mab-5* is no longer expressed in the lineage (Harfe et al., 1998b). The M lineage phenotype of *hlh-8 (nr2061)* animals resembled the *mab-5 (e1239)* phenotype because early division planes of the M mesoblast are aberrant in both mutants (Table 4). We are uncertain at this point of the direct consequences for the division plane defects in the *hlh-8 (nr2061)* or in the *mab-5 (e1239)* worms, but we speculate that CeTwist may be required for cells to respond to dorsal/ventral and left/right cues in the posterior of the worm since the M descendants are often found only on the ventral side instead of in 4 quadrants. In addition, *mab-5 (e1239)* animals have extra sex myoblasts due to cell fate transformations of coelomocytes and some body wall muscles (Table 4). The extra SMs in some *hlh-8 (nr2061)* animals may be due in part to similar transformations. However, in *mab-5* mutant animals, the SM descendants can differentiate and these animals have extra vulval muscles and are capable of laying their eggs (Harfe et al., 1998b; J. Liu and A. F., unpublished). The competence for differentiation in these cells is most likely due to expression of *hlh-8* late in the M lineage in *mab-5 (e1239)* animals. Although some *mab-5* defects can be explained by a lack of CeTwist in the early M lineage, another *mab-5* target must be functioning

Table 4. Summary of M lineage defects in known mutants

	Phenotypes				
Allele	Early M divisions	Vulval muscles	Sex myoblasts	Postembryonic coelomocytes	Body wall muscles
hlh-8 (nr2061)	Variable defects in division planes	No WT muscles	~5% have fewer ~60% have extra	~25% animals have fewer	~70% have fewer ~20% have extra
hlh-1 (cc561)·‡	Dorsal descendants have extra divisions	Extra	2-5 extra SMs	~90% have none	Variable decrease in number due to bwm→SM transf.
mab-5 (e1239)§	Variable defects in division planes	Extra	Extra due to cc→SM and bwm→SM transformations	100% have none	Variable decrease in number due to bwm→SM transf.
lin-12 (0)·¶	Not affected	None	None due to SM→cc transformation	2 extra due to SM→cc transformation	NA**
lin-12 (d)¶	Not affected	Extra	2 extra due to cc→SM transformation	None due to cc→SM transformation	NA

*Data from Harfe et al., 1998a.
‡Hypomorphic allele; *hlh-1 (cc450)* null animals die as early larvae.
§Data from Harfe et al., 1998b.
¶Data from Greenwald et al., 1983; *lin-12 (0)* = null alleles; *lin-12 (d)* = dominant (gain of function) alleles.
**NA = data not available.

in this lineage because *mab-5 (e1239)* animals never make M-derived coelomocytes whereas *hlh-8 (nr2061)* animals often do (Table 4).

Another gene product that functions in the M lineage is CeMyoD (encoded by the *hlh-1* gene). Twist has been reported to antagonize the function of MyoD through titration of partner proteins and by direct interactions (Hebrok et al., 1994; Rohwedel et al., 1995; Spicer et al., 1996; Hamamori et al., 1997; Anant et al., 1998). Our results do not support an antagonistic relationship between CeTwist and CeMyoD in the M lineage. In fact, loss of *hlh-1*function causes a supernumerary SM phenotype similar to that seen in *hlh-8 (nr2061)* animals (Harfe et al., 1998a; Table 4). The division plane defects in the M lineage of *hlh-8 (nr2061)* animals occurred prior to detectable *hlh-1* expression in the M lineage. As noted above, these division plane defects often misposition early M descendants. One interesting hypothesis is that these misplaced cells may no longer be responsive to positional cues that influence *hlh-1* expression and their cell fate.

Evidence also exists for Twist acting as an inhibitor to myogenesis in tissue culture systems by antagonizing other bHLH factors such as MyoD both in vivo and in vitro (Hebrok et al., 1994; Rohwedel et al., 1995; Spicer et al., 1996; Hamamori et al., 1997; Anant et al., 1998). Such experiments, combined with the pattern of *twist* expression, suggest that Twist is required to maintain myoblasts in a proliferative state. Consistent with this view is the *D. melanogaster* expression pattern that shows a decrease in Twist protein prior to differentiation (Bate et al., 1991; Bate, 1993; Baylies and Bate, 1996). However, Baylies and Bate (1996) demonstrated in flies by overexpressing *twist* that a decrease in protein quantity is not required for normal differentiation. Our results also demonstrate that CeTwist is not required for retaining myoblasts in a proliferative state. This conclusion suggests that the observed decrease in *hlh-8::gfp* prior to differentiation observed in *C. elegans* (Harfe et al., 1998b) is not required to allow normal differentiation to proceed.

Harfe and colleagues (1998b) have observed that the FGF receptor homolog *egl-15* and the NK homeodomain transcription factor *ceh-24* are likely to be direct targets of CeTwist based on several pieces of evidence: promoter regions upstream of these genes contain canonical Twist-like E boxes (the signature sites for bHLH protein binding), induction of *hlh-8* expressed from a heat shock promoter turns on *gfp* reporters containing *egl-15* and *ceh-24* promoter sequences, and RNA interference using sequences from *hlh-8* causes a reduction in expression from the same *gfp* reporters. Our data confirms and extends these observations by showing that a deletion in *hlh-8* causes a lack of reporter expression of these targets that is rescued with the addition of wild-type CeTwist. *egl-15* is involved in the migration of SMs to the gonad (Stern and Horvitz, 1991). The lack of *egl-15* expression could contribute to the posterior SM placement and conceivably a subset of developmental defects in *hlh-8 (nr2061)* animals. *ceh-24* mutants have no detectable phenotype and are not constipated (Harfe and Fire, 1998). Therefore, in the enteric muscles there must be at least one other gene that requires CeTwist for its expression. The potential discovery of other genes regulated by CeTwist is likely to reveal a clearer picture of how these gene products are cooperating to contribute to mesoderm development.

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