The FoxF/FoxC factor LET-381 directly regulates both cell fate specification and cell differentiation in C. elegans mesoderm development

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SUMMARY

Forkhead transcription factors play crucial and diverse roles in mesoderm development. In particular, FoxF and FoxC genes are, respectively, involved in the development of visceral/splanchnic mesoderm and non-visceral mesoderm in coelomate animals. Here, we show at single-cell resolution that, in the pseudocoelomate nematode C. elegans, the single FoxF/FoxC transcription factor LET-381 functions in a feed-forward mechanism in the specification and differentiation of the non-muscle mesodermal cells, the coelomocytes (CCs). LET-381/FoxF directly activates the CC specification factor, the Six2 homeodomain protein CEH-34, and functions cooperatively with CEH-34/Six2 to directly activate genes required for CC differentiation. Our results unify a diverse set of studies on the functions of FoxF/FoxC factors and provide a model for how FoxF/FoxC factors function during mesoderm development.

KEY WORDS: FoxF, FoxC, let-381, Forkhead domain, ceh-34, SIX2 homeodomain, eya-1, Eyes absent, Mesoderm, Cell fate specification, M lineage, Coelomocyte, Asymmetry, C. elegans

INTRODUCTION

Proper animal development requires that each cell precisely integrate spatial, temporal and lineal information for its proper specification and differentiation. Dissecting the underlying regulatory networks that govern these processes requires the identification of key transcription factors and their direct target genes. Previous studies in Drosophila and vertebrates have identified a number of transcription factors required for mesoderm development, among them the forkhead domain-containing transcription factors FoxF and FoxC. FoxF1 and FoxF2 are both required for differentiation of the lateral plate mesoderm and proper gut muscle development in mice (Mahlapuu et al., 2001a; Mahlapuu et al., 2001b; Ormestad et al., 2004; Ormestad et al., 2006), whereas the FoxF protein Biniou functions to specify and direct differentiation of visceral mesoderm in Drosophila (Zaffran et al., 2001; Jakobsen et al., 2007; Zinzen et al., 2009), and FoxF in Ciona intestinalis is required for the migration of heart precursors (Beh et al., 2007; Christiaen et al., 2008). In vertebrates, FoxC proteins are expressed in the developing paraxial and intermediate mesoderm, and play important roles in the development of somites, kidneys and the cardiovascular system (Winnier et al., 1999; Kume et al., 2004; Ormestad et al., 2006). Interestingly, Drosophila and vertebrates have at least one homolog of each FoxF and FoxC, whereas the pseudocoelomate nematode C. elegans has a sole FoxF-related factor, LET-381, which is also the closest match for FoxC (Carlsson and Mahlapuu, 2002). In this study, we investigated the role of LET-381/FoxF in the C. elegans postembryonic mesoderm.

The C. elegans postembryonic non-gonadal mesoderm (the M lineage) is derived from a single pluripotent progenitor cell, the M mesoblast. During postembryonic development, the M mesoblast divides reproducibly and characteristically to produce fourteen striated body wall muscles (BWM), two non-muscle coelomocytes (CCs), and two sex myoblasts (SMs) that are precursors of sixteen non-striated egg-laying muscles (Fig. 1A) (Sulston and Horvitz, 1977). The SMs are descendants of the ventral M lineage, whereas the CCs are dorsally derived. The distinction between the dorsal and ventral M lineage is due to the LIN-12/Notch pathway acting on the ventral lineage, and the Sma/Mab TGFβ pathway being antagonized in the dorsal M lineage by the Schnurri homolog SMA-9 (Greenwald et al., 1983; Foehr et al., 2006; Foehr and Liu, 2008). Within the dorsal M lineage, three M lineage intrinsic factors, HLH-1, FOZI-1 and MAB-5, are required for specifying both the BWMs and the CCs (Harfe et al., 1998a; Harfe et al., 1998b; Liu and Fire, 2000; Amin et al., 2007). The difference between BWMs and CCs is due to the presence of a CC-specifying factor, the Six2 homeodomain protein CEH-34, in the undifferentiated CC cells (Amin et al., 2009). We have previously shown that the proper expression of ceh-34 in the undifferentiated CC cells is due to the combination of differential POP-1 (TCF/LEF) transcriptional activity along the anteroposterior axis and the presence of a CC competence factor(s) (Fig. 1B).

In this study, we show that the sole FoxF/FoxC-related protein in C. elegans, LET-381, is a CC competence factor. LET-381/FoxF directly activates ceh-34 expression, and functions synergistically with CEH-34 to promote M-derived CC fate specification. In addition to its role in specifying the CCs, LET-381/FoxF also directly activates the expression of several genes required for differentiation and function of the CCs. Our studies demonstrate at single-cell resolution that LET-381 functions in a feed-forward mechanism to directly regulate both fate specification and differentiation. These findings unify a diverse set of studies on the functions of FoxF/FoxC factors and provide a model for how FoxF/FoxC factors function during mesoderm development.

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MATERIALS AND METHODS

C. elegans strains

Strains were maintained and manipulated using standard conditions (Brenner, 1974). Analyses were performed at 20°C, unless otherwise noted.

The strains LW0683[rps-3(pk1426) II; cels4438 (intricss CC::gfp):III; ays2(ts15g1:–5:cef) IV; avy6(hh-8:–gfp) X] J, LW1066[h16:–lmp:–kmpF] (Jiang et al., 2008) and LW1734[js1475(myo-3::–rgfp) I; rps-3(pk1426) II; cels4438 (intricss CC::gfp):III; ays2(ts15g1:–5:cef) IV; avy6(hh-8:–gfp) X] (Amin et al., 2009) were used to visualize M lineage cells in RNAi experiments. Intricss CC::gfp is a twisted-derived coelomocyte marker, whereas secreted CC::gfp is another coelomocyte marker using a myo-3::–secreted GFP that is secreted from the BWMs and taken up by differentiated Ccs (Harfe et al., 1998a; Harfe et al., 1998b). Additional M lineage-specific reporters were as described by Kostas and Fire (Kostas and Fire, 2002). Other mutations were used: LG X, sma-9(cc604) (Foe et al., 2006); LG III, let-381(gk302); let-381(tm288) (gift of Shohei Mitani, Tokyo Women’s Medical University, Tokyo, Japan); lin-12(n676930tts); lin-12(n6941) (Greenwald et al., 1983; Sundaram and Greenwald, 1993); LG V, ceh-34 (m3733) (Amin et al., 2009).

Plasmid constructs and transgenic lines

pNMA90 (3.9 kb ceh-34::ceh-34 genomic ORF::gfp::unc-54 3UTR) and pNMA94 (3.9 kb ceh-34::ceh-34 ORF::gfp::ceh-34 3UTR) showed identical expression patterns (Amin et al., 2009). pNMA94 was used to generate a series of promoter deletion constructs (Fig. 2A) and constructs carrying mutations in the putative FoxF-binding sites (Fig. 3A).

A 348-bp fragment of the ceh-34 promoter (~2260 to ~1912) excised from pNMA94 was cloned into L3135 of the Fire Lab Vector Kit (http://www.addgene.org) to generate pNMA118 (E1::pes-10Ap::gfp::lacZ::unc-54 3UTR). Corresponding E1 fragments with the putative FoxF-binding sites mutated were excised from the plasmids pNMA116, 124 and 127 to generate plasmids pNMA132, 136 and 137, respectively.

Plasmid for let-381 (RNAi) was obtained from the Ahringer RNAi library (Kamath et al., 2003) provided by Geneservice and was confirmed by sequencing. Fragments spanning 5 kb of the let-381 promoter or the entire coding region were PCR amplified from N2 genomic DNA using iProof High-Fidelity DNA Polymerase (Bio-Rad). These PCR fragments were used to generate the let-381 reporter construct pNMA99: let-381::gfp::ceh-34 genomic ORF::lmp::gfp::unc-54 3UTR. A let-381 cDNA clone, yk76912, which spans the entire ORF and the 3’UTR of let-381, was used to make the forced expression constructs pNMA85 (hsp-16::let-381 cDNA::let-381 3UTR) and pNMA86 (hsp-16::let-381 cDNA::let-381 3UTR). Plasmid pF95 (twist-derived intrinsic CC::gfp) (Harfe et al., 1998b; Kostas and Fire, 2002), phD43 (unc-122p::gfp) (Loria et al., 2004), phD53 (cup-4p::gfp) and phD195 (que-26p::gfp) (Patton et al., 2005) were used for site-directed mutagenesis to generate plasmids depicted in Fig. 6F-I.

All plasmids were verified by sequencing. Transgenic lines were generated using the plasmids pRF4 (Mello et al., 1991) or mec-7::rfp (Amin et al., 2009) as markers.

Heat-shock experiments

LW2500 and LW2502[jls250] and jls250(hh-8:hp::ceh-34+hsp-6p:–5:CEF4) I; cels4438(intricss CC::gfp) III; ays2(ts15g1:–5:cef) IV; avy6(hh-8:–gfp) X] were scored during subsequent larval development for the number and type of M lineage-derived cells.

Antibody production and immunofluorescence staining

Plasmid pNMA70 was used to generate GST-LET-381 (amino acids 179-320) fusion proteins in BL21(DE3) cells. GST-LET-381 fusion proteins were bound to glutathione sepharose 4B beads (Amersham Biosciences) and cleaved from GST by GST-3CPro precision protease (Amersham Biosciences). Soluble LET-381 was extracted and further purified by SDS-PAGE. Gel slices containing purified LET-381 protein were used to immunize rats (Cocalico Biologicals, PA, USA). Resulting antisera CUMC-R27 and CUMC-R28 were used for affinity purification against GST-LET-381 bound to a nitrocellulose membrane (Olmsted, 1981; Smith and Fisher, 1984).

Animal fixation, immunostaining, microscopy and image analysis were performed as described previously (Amin et al., 2007). Guinea pig anti-FOZI-1 (Amin et al., 2007) (1:200), goat anti-GFP (Rockland Immunochemicals; 1:5000), rat anti-LET-381 (1:50) and rabbit anti-HLH-1 (Harfe et al., 1998a) (1:200) antibodies were used.

Electrophoretic mobility shift assays

A let-381 CDNA fragment containing amino acids 1 to 320 was cloned into the pQE expression system (Qiagen) to generate pNM76. pNMA76 was transformed into M15[pREP4] cells for subsequent purification of 6×His::LET-381 fusion proteins under denaturing conditions (Qiagen). Proteins were then re-natured in 1 M Tris pH 8.0 at 4°C. Both the full length and a truncated form of the fusion protein were detected after SDS-PAGE by Coomassie staining and western blot analysis. NMA81 contains the full-length ceh-34 cDNA (Amin et al., 2009) in the pGEX expression system (Amersham). GST-CEH-34 fusion proteins were purified and cleaved using the same method as GST-LET-381 (see above). Soluble CEH-34 protein was used for gel shift reactions. Probe DNA oligos were 3’-end labeled with biotin (Biotin 3’ End DNA Labeling Kit, Pierce). Gel-shift reactions and detection were performed using the LightShift Chemiluminescent EMSA Kit (Pierce).

RESULTS

A 348-bp enhancer element is necessary and sufficient for ceh-34 expression in M lineage-derived CCs

The Six2 homolog ceh-34 is transiently expressed in the undifferentiated M lineage-derived CCs and is required for their proper specification (Amin et al., 2009) (Fig. 1B). Using two ceh-34 reporter constructs, pNMA90 and pNMA94 (see Materials and methods), we have previously shown that the 3.9-kb ceh-34 promoter contains all of the cis-elements required for ceh-34 expression in the M lineage (Amin et al., 2009). To identify the minimal sequences within this 3.9-kb fragment, we generated a series of promoter deletion constructs using pNMA94 (Fig. 2A) and tested the expression of reporter genes in the M lineage. As shown in Fig. 2A, deleting a 348-bp element (~2260 to ~1912) located approximately 2 kb upstream of the ceh-34 open reading frame (ORF) abolished the M lineage expression of the reporter, suggesting that the sequences located within this 348-bp element are necessary for M lineage expression of the gfp::ceh-34 cDNA (Fig. 2A). When placed upstream of a basal promoter (referred to as E1::pes-10Ap::gfp), this 348-bp element (E1) is sufficient to drive reporter expression in the M lineage in a pattern identical to that of the functional gfp::ceh-34 translational fusions (Fig. 2A-D). Thus, the E1 element is both necessary and sufficient for ceh-34 expression in the M lineage.

Two putative FoxF-binding sites in the ceh-34 M lineage enhancer are required for the proper expression of ceh-34 in the M lineage

To identify potential trans-acting factors that might regulate ceh-34 expression via the 348-bp E1 enhancer, we compared the sequence of this enhancer with its corresponding sequences in three closely related Caenorhabditis species, C. briggsae, C. brenneri and C. remanei, and searched for putative transcription factor-binding sites using the TESS software (Schug, 2008). There was a high degree of conservation in the second part of the E1 enhancer, which contains putative binding sites for MyoD, Hox, PBC and Meis transcription factors (Fig. 2E). C. elegans homologs for each of these factors, hhl-1 (MyoD), mab-5 (Hox), ceh-20 (PBC) and unc-62 (Meis) have
been shown to function in M-derived CC specification (Harfe et al., 1998a; Liu and Fire, 2000; Jiang et al., 2009). Furthermore, hlh-1 and mab-5 are known to function upstream of ceh-34 in the M lineage (Amin et al., 2009).

In addition to these sites, the E1 enhancer also contains two adjacent, conserved putative FoxF (ATAAA(C/T)A) binding sites, which we named as FoxF sites 1 and 2 (Fig. 3A) (Peterson et al., 1997). These sites are intriguing as we have also identified a forkhead transcription factor, let-381, in an RNAi screen for transcription factors important for M lineage development (Amin et al., 2009) (see below). Deleting a 21-nucleotide region containing both sites 1 and 2 in the context of the intact ceh-34 promoter [pNMA116 (fkh/H9004; ceh-34p::gfp::ceh-34::ceh-34 3’UTR)] led to the loss of reporter expression in the M-derived CCs without affecting reporter expression in other cell types, such as those in the head (Fig. 3A). Deleting both sites in the E1 enhancer also resulted in the loss of M lineage expression of the E1::pes-10p::gfp reporter (data not shown). To directly test the importance of each of the two putative FoxF-binding sites, we made both deletions and clustered mutations in site 1, site 2, or both sites 1 and 2 in the context of the intact ceh-34 promoter and in the E1 enhancer. Mutations in site 2, but not site 1, completely abolished reporter expression in the M lineage (Fig. 3A). Interestingly, at a very low frequency (3.7%, n=109), mutations in site 1 led to transient ectopic expression of the reporter in the posterior sister cells of M-derived CCs [M.d(l/r)pp; data not shown]. These results demonstrated that the two putative FoxF sites, primarily site 2, are crucial for proper ceh-34 expression in the M lineage.

To further determine the functional significance of these two putative FoxF sites, we deleted them in the ceh-34 promoter and assayed the ability of the ceh-34 transgene driven by the mutant promoter to rescue ceh-34 mutant defects. ceh-34(tm3733) animals are 100% lethal at the L1 stage. As seen in ceh-34(RNAi) animals (Amin et al., 2009), these arrested larvae do not have any functional CCs and exhibit a phenotype similar to CC-uptake mutants, in which a GFP secreted from the BWMs...
LET-381/FoxF is required for CC fates and ceh-34 expression in the M lineage

C. elegans has a single factor encoded by let-381 that is closely related to FoxF and FoxC in other animals (Carlsson and Mahlapuu, 2002). As let-381 mutations lead to embryonic and larval lethality, we used feeding RNAi to investigate the role of let-381 during postembryonic development. Similar to ceh-34(RNAi), let-381(RNAi) resulted in a loss of M-derived CCs (Fig. 4A-D). By following the M lineage in let-381(RNAi) animals using hlh-8::gfp and anti-FOZI-1 immunostaining (Amin et al., 2007), we found that instead of becoming two CCs, M.dlpa and M.drp in let-381(RNAi) animals adopt the fate of their ventral counterparts (M.vlpa and M.vrpa), each giving rise to a SM and a BWM (Fig. 4B). The ectopic SMs migrated to the vulva and divided to produce extra sex muscles (as visualized by egl-15::gfp; Fig. 4D).

Two deletion alleles of let-381, gk302 and tm288, also displayed M lineage phenotypes similar to let-381(RNAi) animals. Although the majority of gk302 and tm288 homozygous mutants are embryonic lethal without any functional embryonic CCs, some survive to become sterile adults. These sterile animals lack functional embryonic and M-derived CCs (visualized by a myo-3p::secreted GFP CC marker) and have extra egl-15::gfp-positive sex muscles (Fig. 4E,F). Thus, like ceh-34, let-381 is required for the specification of M-derived CCs.

LET-381/FoxF expression in the M lineage precedes ceh-34 expression

To determine the expression pattern of let-381, we first generated and affinity-purified anti-LET-381 antibodies and immunostained larvae and embryos. We also generated transgenic lines that expressed a functional let-381::gfp fusion (pNMA99) that rescued the lethality and M lineage defects of let-381(tm288) animals (Fig. 5A; data not shown). Both let-381::gfp and anti-LET-381 immunostaining showed similar let-381 expression patterns, which begin during embryogenesis and persist in a small number of cells throughout larval development (Fig. 5B-D). As expected of a transcription factor, LET-381 localizes to the nucleus (Fig. 5B-N). Double-labeling experiments using the let-381::gfp fusion or anti-LET-381 with anti-FOZI-1 staining (to label M lineage cells) (Amin et al., 2007) showed that LET-381 is transiently expressed in the M lineage and is first detected in M.dlp and M.drp as they begin to divide (Fig. 5E-H). Expression continued in both daughters of these lineage and is first detected in M.dlp and M.drp as they begin to divide (Fig. 5E-H). Expression continued in both daughters of these
and 2 abolished the shift (Fig. 3J). The gel mobility shift was observed with a 381–DNA complex (Fig. 3I), whereas mutating both FoxF sites 1 and 2 in the EMSA assay (Fig. 3J). These data suggest that LET-381 bound to their ability to bind to purified recombinant LET-381 protein in vitro. M lineage enhancer are sites for LET-381 binding, we tested this by electrophoretic mobility shift assay (EMSA). LET-381 bound to the promoter region of the let-381 gene, which is consistent with the in vivo data showing that site 2 is crucial for LET-381 binding in the M lineage (Fig. 3J). Thus, LET-381 might directly regulate ceh-34 expression in the M lineage by directly binding to these FoxF sites. Interestingly, mutation of FoxF site 1 alone completely abolished the gel shift but mutation of FoxF site 2 only slightly reduced the amount of gel shift (Fig. 3I). Furthermore, site 1 mutant oligonucleotides were better competitors than site 2 mutant oligonucleotides in the EMSA assay (Fig. 3J). These data suggest LET-381 has a higher affinity for the second FoxF-binding site, which is consistent with the in vivo data showing that site 2 is crucial for ceh-34 expression in the M lineage (Fig. 3A).
M.d/(l/r)pa to become CCs (Fig. 1). As LET-381 is transiently localized in M.d/(l/r)pp and M.d/(l/r)pa, we tested whether LET-381 might be one of these cofactors. We forced the expression of let-381 using the hls-8 promoter (throughout the M lineage) and the heat-shock-inducible hsp-16 promoter. Forced expression of let-381 alone, using either approach, did not affect M lineage development, nor did it affect the expression pattern of ceh-34 within the M lineage (data not shown), suggesting that other factors are required with LET-381 to activate ceh-34 expression in the M lineage. Interestingly, forced expression of let-381, in conjunction with ceh-34 and eya-1 during M lineage development, led to: (1) the production of up to seven M-derived CCs (Fig. 6A-E); and (2) an overall increase in the efficiency of ectopic CC production compared with forced expression of ceh-34 and eya-1 alone (Fig. 6D,E). By following M lineage development in these animals, we found that most, if not all, BWMs derived from M.d can be transformed into CCs upon ectopic expression of let-381, ceh-34 and eya-1 (data not shown). These results suggest that LET-381 functions together with CEH-34 and EYA-1 to induce CC fate.

To further test if let-381 is required for specifying M-derived CC fates in addition to regulating ceh-34 expression, we performed let-381(RNAi) in transgenic animals expressing hls-8p::ceh-34 and hls-8p::eya-1. Whereas control RNAi-treated animals produced two to four M-derived CCs (91.0%, n=145), let-381(RNAi) led to a complete loss of all M-derived CCs, including both normal and ectopic CCs generated in the transgenic line (93.5%, n=123). Thus let-381 is not only required for the expression of ceh-34, but is also required for the specification of CCs in the presence of ceh-34.

**LET-381/FoxF directly regulates the differentiation of M-derived CCs**

Because LET-381 and CEH-34 together are potent in specifying M-derived CCs, we next tested whether these factors have a direct role in proper differentiation of the CCs. Three genes, unc-122, cup-4 and lgc-26, have been shown to be expressed in all six differentiated CCs (Patton et al., 2005). In addition, a CC-specific enhancer element is located close to the hls-8 promoter (Harfe et al., 1998b). Because LET-381 and CEH-34 together are potent in specifying M-derived CCs, we next tested whether these factors have a direct role in proper differentiation of the CCs. Three genes, unc-122, cup-4 and lgc-26, have been shown to be expressed in all six differentiated CCs (Patton et al., 2005). Moreover, cup-4 and lgc-26 are required for the proper function of the CCs (Patton et al., 2005). In addition, a CC-specific enhancer element is located close to the hls-8 promoter (Harfe et al., 1998b). This 146-bp enhancer is not only required for the expression of hls-8, but when placed upstream of a basal promoter, can drive GFP expression exclusively in all six CCs. We searched for putative LET-381/FoxF [ATAACA (Peterson et al., 1997)] and CEH-34/Six2 [TCAGGTT (Hu et al., 2008) or TGATAC, (Noyes et al., 2008)] binding sites in these promoters and found that unc-122, cup-4 and
lgc-26 all possess at least one putative LET-381/FoxF site within 1 kb upstream of the coding sequence and that cup-4, lgc-26 and hlh-8 also contain putative CEH-34/Six2 sites (see below; Fig. 6F-I).

The 108-bp sequence immediately upstream of the unc-122 coding region is sufficient to drive reporter expression exclusively within all six CCs (Loria et al., 2004). We identified one putative FoxF-binding site in this region and tested reporter expression from both the wild-type and mutant forms of this sequence. As shown in Fig. 6F, mutating this putative FoxF site in the unc-122 promoter led to a loss of GFP expression in both embryonic and M-derived CCs.

The 2.3-kb cup-4 promoter has three putative FoxF-binding sites and two putative Six2-binding sites (Fig. 6G). Mutating all three FoxF sites led to a loss of GFP expression in the M-derived CCs and fainter GFP expression in the embryonic CCs (Fig. 6G,J,K). Although mutating both Six2-binding sites did not affect GFP expression in the CCs (Fig. 6G,L), mutating both Six2 sites and all three FoxF sites together led to a complete loss of GFP in both M- and embryonically derived CCs (Fig. 6G,M).

The 957-bp lgc-26 promoter contains three putative FoxF sites and one putative Six2 site (Fig. 6H). Mutating all three FoxF sites or the Six2 site separately did not affect GFP expression in M- or embryonically derived CCs. However, mutating all four sites together led to reduced GFP expression in all six CCs (Fig. 6H).

The 146-bp enhancer element upstream of hlh-8 does not contain any putative FoxF site, but does have a single putative Six2 site. Mutating this putative Six2 site led to the loss of GFP expression in all CCs of wild-type animals (Fig. 6I).

The putative CEH-34/Six2-binding sites in the above CC enhancers are of two distinct types: TCAGGGTT (Hu et al., 2008) in the cup-4 and lgc-26 promoters; or TGATAC (Noyes et al., 2008) in the hlh-8 enhancer (Fig. 6G-I). We tested in vitro the ability of CEH-34 to bind to each type of Six2 site. As shown in Fig. 6N, CEH-34 protein resulted in the mobility shift of oligonucleotides centered around both types of Six2-binding site (NMA-329/330 and NMA-325/326). This mobility shift was abolished when the Six2 site in the cup-4 promoter was mutated (NMA-331/332), and was significantly reduced when the Six2 site in the hlh-8 enhancer was mutated (NMA-327/328), suggesting that CEH-34 can bind to both sites. Competition assays further showed that the wild-type oligonucleotide NMA-325/326 was a more potent competitor than the mutant oligonucleotide NMA-327/328 (Fig. 6O). Furthermore, NMA329/330 was a more potent competitor than NMA-325/326 (Fig. 6O), suggesting that CEH-34 has a higher affinity to TCAGGGTT than to TGATAC. Thus, CEH-34 can bind to two distinct sets of sequences, albeit at different affinities.

We performed an in silico search for putative FoxF- and Six2-binding sites in the S’ regions of 14 additional genes that are expressed in and required for the function of differentiated CCs (Nonet et al., 1999; Fares and Greenwald, 2001b; Grant et al., 2001; Hwang and Horvitz, 2002; Xue et al., 2003; Dang et al., 2004; Roudier et al., 2005; Sato et al., 2005; Chen et al., 2006; Gengyo-Ando et al., 2007; Chun et al., 2008; Sato et al., 2008; Amin et al., 2009; Schwartz et al., 2010). Since the FoxF- and Six2-binding sites for cup-4 and lgc-26 are all clustered immediately upstream of the translation initiation codon (Fig. 6), we focused our search on the 1.5-kb sequences upstream of the ATG. Consistent with our hypothesis that CEH-34 and LET-381 directly regulate the expression of CC differentiation factors, nine out of the 14 genes (mtm-9, rab-10, vps-45, rme-1, rme-6, vps-27, unc-11, sqv-1, hmt-1) had FoxF- or Six2-binding sites in their 5’ regions. Conversely, of 13 randomly selected differentiation genes in the gut (McGhee et al., 2009), where ceh-34 and let-381 are not expressed, only three contained a putative FoxF- or Six2-binding site (data not shown). Taken together, our results suggest that LET-381 and CEH-34 differentially, but probably directly, regulate CC-specific factors for proper CC differentiation and function.

**DISCUSSION**

**LET-381/FoxF directly regulates the CC specification gene CEH-34/Six2 and downstream target genes**

Our data demonstrate that LET-381/FoxF directly regulates the expression of another highly conserved transcription factor, CEH-34/Six2, during C. elegans postembryonic mesoderm development: (1) let-381 and ceh-34 are both required for CC fates; (2) let-381 expression precedes, and is required for, ceh-34 expression in the M lineage; (3) the two putative FoxF-binding sites in the ceh-34 promoter are essential for ceh-34 expression in the M lineage; and (4) recombinant LET-381 protein binds to these sites in vitro. Interestingly, FoxF site 2 (Fig. 3A) is absolutely required for ceh-34 expression, whereas mutations in FoxF site 1 lead to a low level of ectopic expression of ceh-34 in the M lineage. Site 1 might represent a binding site for another transcription factor that helps to prevent ceh-34 expression in the posterior sisters of M-derived CCs, such as the factor that has been hypothesized to function downstream of the TCF/LEF homolog POP-1 (Amin et al., 2009).

We have previously shown that forced expression of ceh-34 and its cofactor eya-1 in the M lineage results in a modest increase in the number of M-derived CCs (Amin et al., 2009). let-381(RNAi) in these animals leads to a complete loss of both endogenous and ectopic M-derived CCs, suggesting that let-381 is not only required for ceh-34 expression, but also for CC specification in the presence of ceh-34. Interestingly, forced expression of let-381, ceh-34 and eya-1 throughout the M lineage results in the transformation of all presumptive BWMs in the dorsal M lineage to CCs (Fig. 6; data not shown), suggesting that LET-381 functions together with CEH-34 and EYA-1 to induce CC fate. It is intriguing that only dorsal M lineage descendants are affected in animals with forced expression of let-381, ceh-34 and eya-1. Dorsoventral patterning of the M lineage is under the control of two independent signaling pathways; the LIN-12/Notch pathway is required for ventral M lineage fates, whereas inhibition of the Sma/Mab TGFβ pathway by SMA-9 is required for dorsal M lineage fates (Foehr et al., 2006; Foehr and Liu, 2008). The expression of both let-381 and ceh-34 in the M lineage is under the control of both signaling pathways (Amin et al., 2009) (Fig. 5P-S). It is possible that additional targets of these two pathways are required for CC fate specification. These targets could include a pan-dorsal factor that makes dorsal cells competent to respond to the transcriptional activity of LET-381 and CEH-34, and/or a pan-ventral factor that inhibits the function of let-381 and ceh-34. This is not an unreasonable notion as previous studies have found pan-dorsal or pan-ventral BWM localization of the TGFβ-like molecule UNC-129 and the forkhead transcription factor UNC-130, respectively (Colavita et al., 1998; Nash et al., 2000). Consistent with this idea, we found that forced expression of let-381, ceh-34 and eya-1 in a lin-12 loss-of-function mutant led to the ectopic production of M-derived CCs both dorsally and ventrally (data not shown).

The conclusion that LET-381 functions in parallel to CEH-34 in regulating CC specification is supported by our findings that genes specifically expressed and required for CC function require intact FoxF- and Six2-binding sites in their promoters for proper expression. As shown in Fig. 6, mutating the putative FoxF- and Six2-binding sites in the promoters of unc-122, cup-4 and lgc-26, as well as in a CC-specific enhancer found in the hlh-8 promoter,
resulted in either a complete loss of, or reduced, reporter gene expression in the CCs. Moreover, putative FoxF- and/or Six2-binding sites are found in the upstream regions of a number of genes expressed in CCs with a reported role in endocytosis. These findings, coupled with the observation that ceh-34(tm3733) and let-381(tm288 or gk302) null mutant animals have no functional CCs, suggest that LET-381/FoxF and CEH-34/Six2 function combinatorially to directly regulate the differentiation of all six CCs in the worm. Interestingly, FoxF- and/or Six2-binding site mutations in each of the promotors tested resulted in distinct alterations of reporter gene expression, affecting either only M-derived CC expression or expression in both embryonic and M-derived CCs (Fig. 6). It is possible that the local sequence contexts where the putative FoxF- and Six2-binding sites reside help to distinguish between gene expression in the embryonically derived and the M-derived CCs. The observed diversity is consistent with previous findings by Brown et al. (Brown et al., 2007) and Zinzen et al. (Zinzen et al., 2009), and argues against a stringent transcription factor binding code in the cis-regulatory modules regulating CC-specific gene expression.

**A model for the specification of non-muscle CCs in postembryonic mesoderm**

Our findings are consistent with a model in which LET-381 is the key component in a feed-forward gene network that regulates CC specification within the M lineage (Fig. 7). The proper pattern of LET-381 expression is due to the combinatorial actions of: (1) the presence of the M lineage intrinsic transcription factors HLH-1, FOZI-1 and MAB-5; (2) the lack of LIN-12/Notch signaling; and (3) presence of SMA-9, which antagonizes the Sma/Mab pathway. POP-1/SYS-1 asymmetry along the anteroposterior axis affects ceh-34 expression (Amin et al., 2009). However, the let-381 expression pattern was not altered in sys-1(RNAi) and pop-1(RNAi) animals (data not shown). We have previously reported that SYS-1 and POP-1 are asymmetrically localized at both the 8-M and 16-M stages, and that the earliest M lineage defects we observed in sys-1(RNAi), pop-1(RNAi), sys-1(q544), pop-1(q645) and pop-1(q624) animals are all at the 16-M stage (Amin et al., 2009). Because both pop-1 and sys-1 are required for worm viability, we cannot rule out the possibility that POP-1/SYS-1 asymmetry might also be required for the asymmetric distribution of LET-381 in the M lineage.

Once properly localized, LET-381 directly activates CEH-34 expression, and functions together with CEH-34 and its cofactor EYA-1 to promote CC fate specification and differentiation by directly regulating the expression of CC-specific factors (Fig. 7). LET-381 also plays a role in suppressing the ventral SM fate, as let-381(RNAi) results in a CC to SM fate transformation (Fig. 4B). However, the inability of LET-381, CEH-34 and EYA-1 to transform ventral M lineage cells to CCs suggests that other pan-dorsal or pan-ventral factor(s) must also be under the control of the dorsoventral asymmetry pathways. Additionally, the inability of forced expression of LET-381 on its own to induce ceh-34 expression or ectopic CC fates suggests that additional factor(s) are required to restrict ceh-34 expression in the M lineage (Fig. 7). These additional factors might also be targets of Sma-9 and/or FOZI-1, as forced expression of let-381, ceh-34 and eya-1 did not rescue the lack of M-derived CC phenotype of sma-9(cc604) and fozi-1(cc609) mutants (data not shown).

**Evolutionarily conserved roles of FoxF and FoxC transcription factors during mesoderm development**

FoxF transcription factors are important for the development of lateral plate mesoderm, specifically visceral/splanchnic mesoderm, in many organisms, including Drosophila (Biniou), Ciona intestinalis (FoxF), Xenopus (FoxF1) and mouse (FoxF1 and FoxF2). In Drosophila, Biniou is a master regulator of the visceral mesoderm, and functions together with other mesoderm-specific factors to directly regulate multiple genes involved in the specification of the visceral mesoderm and the differentiation of gut muscles (Zaffran et al., 2001; Jakobsen et al., 2007; Liu et al., 2009). Similarly, mice lacking FoxF1 and FoxF2 have improper differentiation of the lateral plate mesoderm and its derivatives, and Xenopus FoxF1 is required for intestinal smooth muscle development (Mahlapuu et al., 2001a; Mahlapuu et al., 2001b; Ormestad et al., 2004; Tseng et al., 2004; Ormestad et al., 2006). In
addition, FoxF is required for heart precursor migration by directly activating the effector gene RhoDF in *Ciona intestinalis* (Beh et al., 2007; Christiaen et al., 2008). Unlike FoxF, FoxC proteins are present in multiple non-visceral mesoderm tissues, including the developing paraxial and intermediate mesoderm, and play important roles in the development of somites, kidneys and the cardiovascular system (Winnier et al., 1999; Kume et al., 2000; Kume et al., 2001; Wilm et al., 2004).

Interestingly, *Drosophila* and vertebrates have a coelom and have at least one homolog each of FoxF and FoxC. *C. elegans* is a pseudocelomate and LET-381 is the only FoxF homolog in *C. elegans* and also the closest match for FoxC (Carlsson and *C. elegans* system (Winnier et al., 1999; Kume et al., 2000; Kume et al., 2001; present in multiple non-visceral mesoderm tissues, including the addition, FoxF is required for heart precursor migration by directly

References


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