ceh-16/engrailed patterns the embryonic epidermis of *Caenorhabditis elegans*

Giuseppe Cassata[1,2], Gidi Shemer[3], Paolo Morandi[2], Roland Donhauser[1], Benjamin Podbilewicz[3] and Ralf Baumeister[1,4,*]

[1]ABI/Molecular Neurogenetics, LMU Munich, 80336 Munich, Germany
[2]IFOM (Firc Institute of Molecular Oncology Foundation), 20139 Milan, Italy
[3]Department of Biology, Technion-Israel Institute of Technology, 32000 Haifa, Israel
[4]BioIII/Bioinformatics and Molecular Genetics, University of Freiburg, Schaenzlestrasse 1, D-79104 Freiburg, Germany
*Author for correspondence (e-mail: baumeister@celegans.de)

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Summary

***engrailed* is a homeobox gene essential for developmental functions such as differentiation of cell populations and the onset of compartment boundaries in arthropods and vertebrates. We present the first functional study on *engrailed* in an unsegmented animal: the nematode *Caenorhabditis elegans*. In the developing worm embryo, *ceh-16/engrailed* is predominantly expressed in one bilateral row of epidermal cells (the seam cells). We show that *ceh-16/engrailed* primes a specification cascade through three mechanisms: (1) it suppresses fusion between seam cells and other epidermal cells by repressing *eff-1/fusogen* expression; (2) it triggers the differentiation of the seam cells through different factors, including the GATA factor *elt-5*; and (3) it segregates the seam cells into a distinct lateral cellular compartment, repressing cell migration toward dorsal and ventral compartments.**

Key words: *C. elegans*, *engrailed*, Patterning

Introduction

The conserved homeodomain transcription factor *engrailed* was first identified in 1926 as a spontaneous recessive mutation in *Drosophila* (Eker, 1929). *engrailed* turned out to be a key gene involved in the development of *Drosophila* appendages and segments, where it specifies the posterior compartment. *engrailed* has therefore been named a 'selector' gene (Garcia-Bellido and Santamaria, 1972; Lawrence and Morata, 1976; Nusslein-Volhard and Wieschaus, 1980). In vertebrates, *En1* is recruited during the development of vertebrate limbs. It is essential for the maintenance of the ventral compartment specification (Loomis et al., 1996) and has a crucial role in patterning the mid-hindbrain boundary (Danielian and McMahon, 1996).

One striking fact is the similarity between the involvement of *engrailed* in the specification of the posterior compartment in the wing imaginal disc of *Drosophila* and in the specification of the ventral ectoderm in the developing limb in mouse. It has been shown that during embryonic development the anterior ectodermal ridge (AER) of the developing limb remarkably resembles the anterior/posterior (A/P) compartment boundary in the fly. Both the A/P boundary and the AER express *decapentaplegic* (*dpp*)/BMP2 homologous proteins of the TGF-β family, which are repressed by *en/EN1* in adjacent cells. Additionally, in both cases the expression of *dpp*/BMP2 is induced by *hedgehog* (*hh*)/Shh from neighboring cells. These neighboring cells in *Drosophila* are the posterior cells, where *hh* depends on *engrailed*. In the mouse limb, Shh is expressed posteriorly adjacent to, and maintained by, the AER, depending indirectly on EN-1 function (reviewed by Hidalgo, 1998). In addition to the regulation of organizers, *engrailed* is required to preserve compartment boundaries in *Drosophila*. When the selector gene *engrailed* is removed, in vivo, from a posterior clone of cells in the wing, those cells gain anterior affinity. They sort out from posterior cells and, if in contact with anterior cells, sort into and mix with them (Lawrence and Struhl, 1982; Morata and Kerridge, 1982). This segregation mechanism might be controlled in part by regulation of cell adhesion molecules (Dahmann and Basler, 1999). It has been suggested that the ancestral function of *engrailed* may be neuronal targeting, because it regulates the connectivity through the transcriptional regulation of cell adhesion molecules in the central nervous system in arthropods and vertebrates (Gibert, 2002; Vincent, 1998). Moreover, *engrailed* has been proposed to play a general role in segmentation of protostomes (Prud'homme et al., 2003).

In *Caenorhabditis elegans*, the functions of *engrailed* have not yet been described. Instead, the function of GATA and other homeobox-containing transcription factors have been studied in the patterning of the epidermis in roundworms. Briefly, the GATA factor ELT-1 specifies general epidermal identity (Page et al., 1997). The epidermis is subsequently patterned in three morphologically distinguishable major areas during embryogenesis: (1) dorsal cells that fuse to form the syncytia hyp6 and hyp7 during embryonic elongation (Podbilewicz and White, 1994), (see Movie 1 in the

supplementary material); (2) two single left and right rows of lateral seam cells; and (3) the ventral P cells whose descendants either fuse postembryonically to hyp6 and hyp7 or develop vulval structures and the ventral nerve cord (Podbilewicz and White, 1994; Sulston et al., 1983). LIN-39/HoxD4/Dfd and CEH-20/Exd play a crucial role in repressing the cell fusion of some posterior descendants of the P cells (Clark et al., 1993; Maloof and Kenyon, 1998; Shemer and Podbilewicz, 2002; Wang et al., 1993). In addition, the operon encoding the two GATA factors ELT-5(=EGL-18) and ELT-6 is important for differentiation/fusion-repression in the lateral seam cells (Koh and Rothman, 2001) and for cell fusion-repression in the vulval precursor cells (VPCs), where it is controlled by LIN-39/HoxD4/Dfd (Koh et al., 2002). A general effector for cell fusion in the epidermis of the worm is the transmembrane protein EFF-1 (Mohler et al., 2002). Moreover, EFF-1 is both necessary and sufficient for epithelial and myoepithelial cell fusion in *C. elegans* (Shemer et al., 2004). It has also been shown that LIN-39 represses the expression of *eff-1* in the VPCs (Mohler et al., 2002; Shemer and Podbilewicz, 2002).

In this study we show how *ceh-16/engrailed* controls the differentiation of the seam cells, thereby patterning the embryonic epidermis of *C. elegans*. *ceh-16/engrailed* represses the fusion of the seam cells with the neighboring epidermal cells by repressing the expression of the fusion effector *eff-1*. *ceh-16/engrailed* also triggers the expression of *elt-5* and other seam cell markers and is indispensable for alae formation (a hallmark of seam cell differentiation). We also show that in the *ceh-16/engrailed* mutant the seam cells lose their lateral position and migrate either dorsally or ventrally, intermingling with these cells. Therefore, seam cells in the wild-type context seem to act by preventing cell migration and maintaining embryonic compartment.

Materials and methods

Cloning of the *ceh-16* cDNA

The *ceh-16* cDNA was cloned by RT-PCR using SMART™ RACE cDNA Amplification Kit protocol (Clontech). As the 5′ UTR contains stop codons in all three reading frames, the methionine in the second exon constitutes the bona fide start methionine. GenBank sequence identifier: AY647457.

Isolation of *ceh-16* mutants

*ceh-16(lg16)*III, *ceh-16(lg17)*III were obtained by screening ethylmethanesulfonate (EMS) mutagenized worm libraries via PCR according to Anderson (Anderson, 1995); breakpoints of the deletions were sequenced twice independently using standard procedures. The deletion in *ceh-16(lg16)* spans 2218 bp and encompasses 560 bp of the promoter region, the transcriptional start and the first four of the five exons. Exon 5 encodes the C-terminal 51 amino acids. By molecular means this allele is predicted to be a null allele. In *ceh-16(lg17)*, the deletion spans 867 bp encompassing 471 bp of the promoter region, the transcriptional start and most of the second exon including the start methionine and the first 56 amino acids. Since the transcription start in *ceh-16(lg17)* is severely compromised and the phenotype is identical in both *ceh-16(lg16)* (Table 1) and *ceh-16(lg17)*, both alleles probably constitute a null allele. Breakpoint for *ceh-16(lg16)*: GATCGAAAAAGTAGTG/CAGTTGTTTTGGCATGA. Breakpoint for *ceh-16(lg17)*: TAATTCCCATGTTATATT/GCACAAGATATTCCGATC. The sequences of the primers used for screening are available upon request. Both mutants were out-crossed ten times prior to analysis.

C. elegans strains

Nematodes were maintained as previously described (Wood, 1988). Strains were kept at 20°C unless otherwise noted. The wild-type strain N2 was used unless otherwise stated. Strains with the following mutant alleles, chromosomal aberrations or transgenic arrays were used in this work: *eff-1(hy21)*II (Mohler et al., 2002), *jcIs1* IV (integrated *ajm-1::gfp* strain), *ced-1(e1735)*I; *unc-119(ed4)* III; *wIs78* [contains pDP#MM016B (*unc-119*+), pJS191 (*ajm-1::GFP*); pMF1 (SCM::GFP – nuclear seam cell marker), and cosmid F58E10], *dpy-20(e2017)*IV, *wIs66* [*elt-5::gfp* containing pKK7 (Koh and Rothman, 2001)], Ex [*eff-1p::gfp*] (Mohler et al., 2002).

Transgenic strains

Transgenic strains were obtained using standard procedures (Mello et al., 1991), adapted as in Cassata et al. (Cassata et al., 2000). *ceh-16::gfp* translational fusions were injected at a concentration of 30 μg/μl along with 50 μg/μl pRF4 (*rol-6* dm) plasmid. Roller lines were crossed into heterozygous *ceh-16(lg16)* and tested for their rescue ability by selecting homozygous transgenics (genotypization by PCR). Thereafter, the rescued strain was crossed into *jcIs1* for microscopical analyses. Transgenic Ex [*wrt-5::gfp*] and Ex [*wrt-2::gfp*] strains were a generous gift from T. Burglin (Aspock et al., 1999). The *wrt-5::gfp* and *wrt-2::gfp* extrachromosomal arrays were integrated as follows: 50 transgenic L4 were irradiated with UV using a Stratalinker (Model 1800) from Stratagene at 30,000 μJ/cm^2. After irradiation the animals were singled. After starvation the plates were chunked to let the worms crawl out of the agar; 250 were singled and analyzed for their ability to produce 100% transgenics in the offspring. One integrated line of each transgenic was isolated in this way. Both were out-crossed twice. Transgenic lines of *nhr-73::gfp* and *nhr-74::gfp* were obtained as described in Miyabayashi et al. (Miyabayashi et al., 1999).

Lethality tests

Candidate young adult heterozygous *ceh-16(lg16)* or *ceh-16(lg17)* P0 animals were singled and grown on agar plates for 4-5 hours (this allowed each worm to lay 15-20 eggs). Thereafter they were removed and the genotype was determined via PCR. The sum of all the eggs (of the positive plates) was counted. The next day, the number of dead eggs was determined. A similar procedure was adopted with *[ceh-16(lg16)/+]X[ceh-16(lg17)/+]* crosses: males derived from a *ceh-16(lg17)* cross with N2 males were crossed with *ceh-16(lg16)* hermaphrodites. These hermaphrodites were used as P0 for the lethality test. The presence of both alleles in the offspring was tested via PCR. Dead eggs were counted as above.

RNAi experiments

The full-length *ceh-16* cDNA was cloned into pBluescript II SK- using *Hin*dIII and *Bam*HI sites. In vitro transcribed ssRNA from linearized vectors was produced using commercially available T3 and T7 RNA polymerase systems (Promega). Annealed dsRNA was injected into young adult hermaphrodites at a concentration of 1 μg/μl. The offspring was analyzed (by microscopy or lethality test as described above). *elt-5(RNAi)* experiments were performed similarly using the F55A8.1 RNAi clone from the MRC *C. elegans* RNAi bank.

Heatshock experiments

The PCR amplified full-length *ceh-16* cDNA was inserted in frame into the *Kpn*I site of the heatshock promoter (*hsp16-2*) contained in construct pPD49.78 (Stringham et al., 1992) and sequenced.

To perform heatshock experiments, two independent lines of transgenic worms were constructed by injecting 5 μg/μl of heatshock promoter (with or without *ceh-16* for control purposes) along with pMH86 (*dpy-20*+) (Han and Sternberg, 1991) into a *dpy-20(e2017)*IV; *wIs66* [*elt-5::gfp*] strain. Plates containing transgenic animals were heatshocked three times for 1 hour at 30°C (recover period between

heatshocks: 1 hour at 15°C). Transgenic offspring L1 was analyzed the next day for ectopic expression of ELT-5::GFP.

Antibody stainings and microscopy

Antibody staining was performed according to Waddle et al. (Waddle et al., 1994). Light microscopy was performed using a Zeiss Axioplan2 imaging microscope, Zeiss Axiocam HRc camera and Axiovision software. Confocal microscopy was performed as in Shemer and Podbilewicz (Shemer and Podbilewicz, 2002), and confocal time-lapse movies were recorded taking Z series projections each 5-10 minutes for 1.5 hours at 20°C (Rabin and Podbilewicz, 2000).

Results

ceh-16/engrailed cDNA cloning and expression

The structure and some functions of *engrailed* are conserved from arthropods to mammals. To study for the first time *engrailed* in an unsegmented animal, the nematode *C. elegans*, we first cloned the cDNA by RT-PCR (see Materials and methods). The encoded protein sequence contains all the critical domains of an *engrailed-like* factor, unlike any other protein encoded in the worm genome (Fig. 1B; and see Fig. 1 in the supplementary material).

The expression of *ceh-16* in *C. elegans* was analyzed using transgenic animals bearing full-length translational *gfp* fusions. This fusion construct rescued the mutant (see below), suggesting that all required promoter elements are represented. Expression is most robust throughout embryonic development (Fig. 1C,D) from 250 minutes after the first cleavage until the early 3-fold stage. Expression was observed in the nuclei of hyp5, H0-H2, V1-V6 and T. These cells constitute a bilateral row of cells called seam cells (Fig. 1G,H). Additional *ceh-16::gfp* expression was also observed in cells of the AB lineage at stages prior to the one described above (28-56 cell stage). These cells were determined in 100 minute embryos as being ABprap, ABarpp, ABpraa, ABplap, ABplaa, ABarpa and ABaraa (not shown). In later embryonic stages we observed expression in anterior neurons (not determined, see Fig. 2 in the supplementary material) and in the DA1 and DD1 motoneurons after hatching.

To further investigate the epidermal expression, an in situ staining with a monoclonal antibody (4D9) that recognizes *engrailed* proteins in many species was performed (Patel et al., 1989). Localized immunoreactivity in the seam cells was detected during the same stages of embryonic development (Fig. 1E,F). This additionally confirms the expression of *ceh-16* in the nuclei of these cells.

In summary, *ceh-16* encodes an *engrailed* homolog in *C. elegans*. *ceh-16* is expressed in the AB lineage during early embryogenesis, in the lateral seam cells and neurons during morphogenesis, and in two motoneurons during postembryonic stages.

ceh-16/engrailed is required in the seam cells during embryonic development

To study the functions of *ceh-16* in vivo, two deletion alleles [*ceh-16(lg16)* and *ceh-16(lg17)*] were isolated from an EMS deletion library. As both mutations delete the transcriptional start and a large part of the coding region, they are predicted to be null alleles (Fig. 1A; see Materials and methods for details). Both mutant alleles are recessive embryonic lethal, do

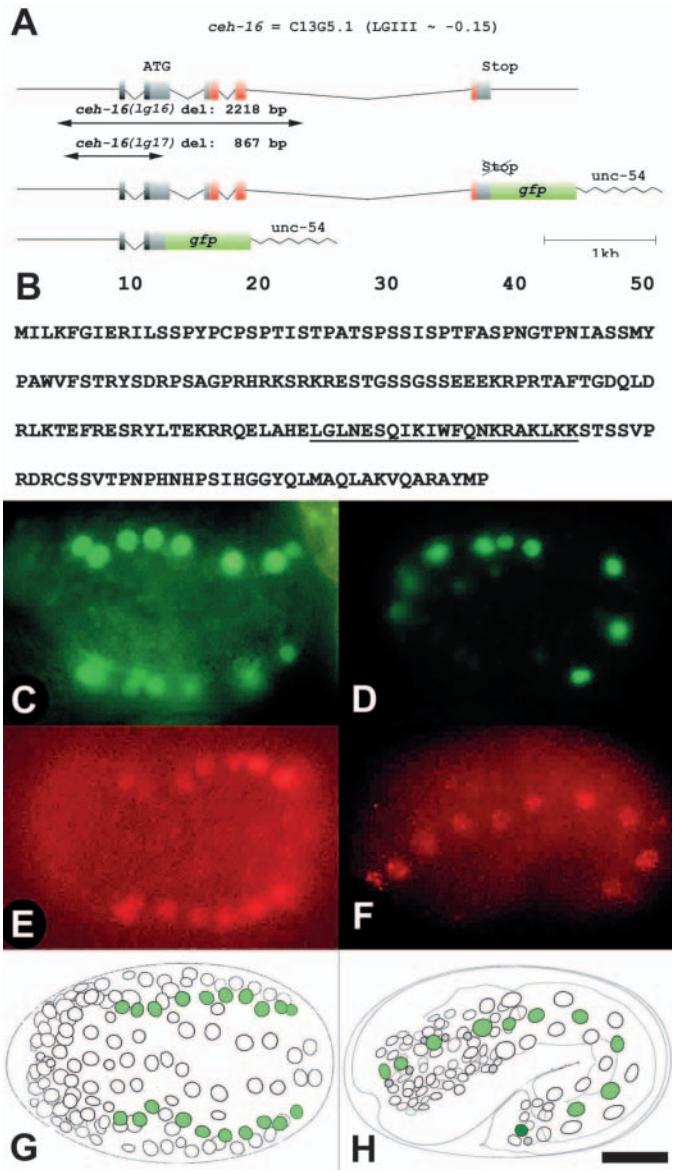

Fig. 1. *ceh-16* structure, mutants and expression pattern. (A) Exon/intron structure of *ceh-16* (C13G5.1) on chromosome III. The *ceh-16* gene is organized in five exons, the start methionine being in the second exon and the stop codon in the fifth exon. Structure of the deletion mutants. Bottom: structure of translational *gfp* fusion constructs used in this study. In both constructs *ceh-16* expression is driven by the endogenous promoter region and contain the *unc-54* 3′ UTR of the plasmid pPD95.75. The construct on top rescued the *ceh-16* phenotype mutant. (B) Amino acid sequence of the proposed *ceh-16* gene product (187 aa), underlined: epitope for Mab 4D9 (Patel et al., 1989). (C,D) Expression pattern of the rescuing *gfp* construct is most robust from 250 minutes after the first cleavage (C) throughout embryonic development (D) (1.5-fold stage of elongation) until early 3-fold stage (not shown). Expression was observed in the nuclei of hyp5, H0-H2, V1-V6, T. Some of these cells are not in the focal plane of the pictures. (E,F) Antibody staining of embryos at the same developmental stages as shown in C,D. All nuclei that expressed *ceh-16::gfp* were also stained with the monoclonal antibody 4D9 (Patel et al., 1989). (G,H) Schematic representation of the position of *ceh-16* expressing cells [based on Sulston et al. (Sulston et al., 1983)]. Scale bar: 10 μm.

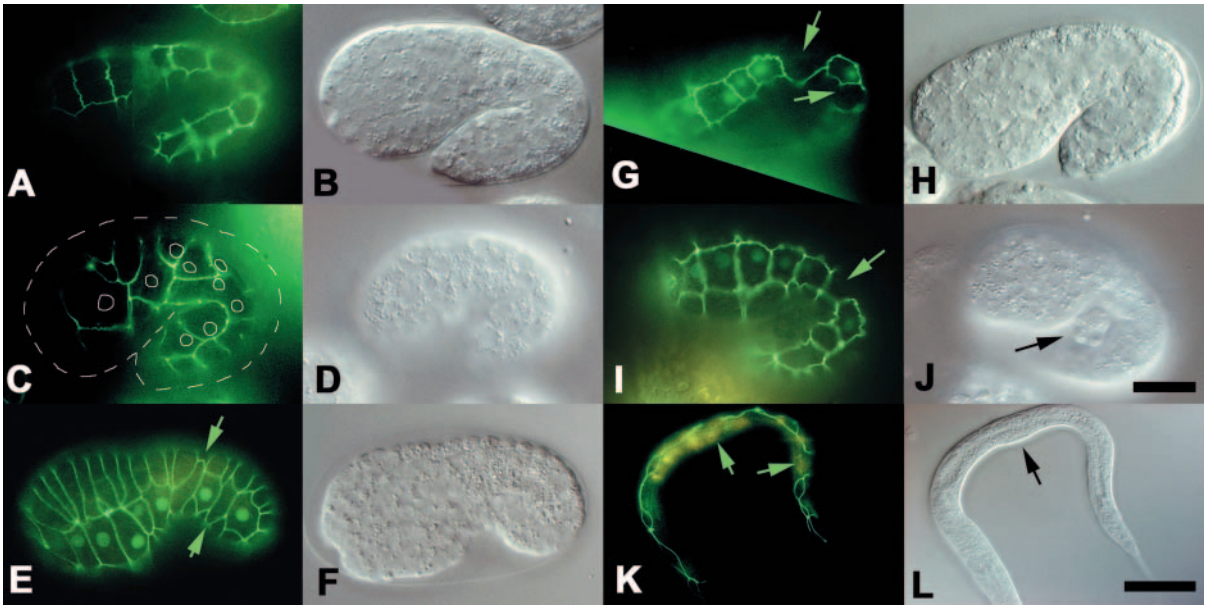

Fig. 2. Phenotype of *ceh-16(lg16)* mutants and mosaic animals. (A,C,E,G,I,K) Fluorescence micrographs of living animals [1.5-fold stage, except for E,F (bean stage) and K,L (L1 larvae)] expressing the adherens junction marker *ajm-1::gfp* in order to visualize cell boundaries in the epithelia. (B,D,F,H,J,L) Corresponding Nomarski pictures. (A,B) Seam cells in wild-type embryos form one line of ten cells (C,D) *ceh-16(lg16)* embryo (approx. same stage) with disorganized epidermal structure. These animals normally do not elongate and do not hatch. The position of seam cell nuclei and the shape of the embryo are sketched. The initiation of ectopic cell fusion events can also be observed. (E,F) Mosaic analysis: Bean-stage *ceh-16(lg16)* embryo rescued with *ceh-16::gfp*. Seam cells that do not express nuclear *ceh-16::gfp* are misshapen and the boundaries intermingle with dorsal and ventral cells (E, arrows). At this stage the dorsal hypodermis has not yet fused to form a syncytium. (G,H) 1.5-fold *ceh-16(lg16)* embryo mosaic for *ceh-16::gfp*. Loss of *ceh-16* results in fusion with the dorsal and ventral hypodermis (arrows). (I,J) Same genotype, one cell fusion event is shown (arrow in I). Leakage of internal cells through the epidermis is shown (arrow in J). (K,L) Mosaic (semi-rescued) L1 larva: fusions are shown (arrows in K). At the same position the larva has a lump (arrow in L). Scale bars: 10 μm for embryos; 20 μm for L1 larvae.

not complement each other and show a very similar phenotype to worms subjected to *ceh-16* RNA interference (RNAi) (Table 1; and see Movie 3 in the supplementary material). As the penetrance of the phenotype of both deletion alleles is identical, we used *ceh-16(lg16)* and *ceh-16(RNAi)* for further experiments. The full-length translational *gfp* construct used for expression studies (Fig. 1A) was sufficient to fully rescue the mutant phenotype (see below), whereas a shorter translational *gfp* construct (Fig. 1A) that contained only the transcriptional start and the first exon did not (data not shown), confirming the specificity of the phenotype.

Microscopic analysis of the *ceh-16(–)* embryos revealed that the epidermal cells were disorganized, causing severe morphological defects and lack of elongation (Fig. 2C,D). To examine what led to this terminal phenotype the rescuing transgene was used as a marker for *ceh-16(+)* cells in a *ceh-16* mutant background. As, in transgenic *C. elegans*, extrachromosomal arrays are frequently lost, embryos that expressed the array in a subset of the seam cells were analyzed (mosaic analysis). We found that, in mosaic animals, seam cells lacking *ceh-16* in their nuclei showed a dorsal and/or ventral displacement with no obvious directional preference. In addition to the positioning defects, the *ceh-16(–)* cells fused to the dorsal or ventral epidermis (Fig. 2G,H; arrows). In some mosaic animals (8/63), mutant seam cells projected ventrally in such a way that may have destabilized ventral closure (George et al., 1998), implying that embryonic lethality may be a result of leakage of internal cells. In Fig. 2I,J we show an example of such an embryo with free undetermined cells near the ventral closure that may have leaked out of the embryo at

Table 1. Zygotic function of *ceh-16*

Genotype of P0	Percentage of dead embryos in the F1	Percentage of L1/F1 with *ceh-16* phenotype†	*n* (P0)
ceh-16(lg16)/+	22 (*n*=601)	0	20
ceh-16(lg17)/+	23 (*n*=589)	0	18
[ceh-16(lg16)/+]X[ceh-16(lg17)/+]	22 (*n*=622)	0	22
*ceh-16(RNAi)**	70 (*n*=437)	30	20
N2 (wild type)	0 (*n*=428)	0	19

Both alleles are recessive lethal. Escapers of *ceh-16(lg16)* and *ceh-16(lg17)* that do not die in the egg shell become amorphous short L1 larvae and do not further develop.

*20 injected animals.

†See Figs 2, 4 and 5 for a description.

the ventral side. In all the mosaic animals, the seam cells expressing CEH-16::GFP [*ceh-16(+)*] were rescued and those lacking CEH-16::GFP [*ceh-16(–)*] showed the phenotype described above (displacement and/or ectopic fusion; Fig. 2E,F). Partial rescued animals manage to hatch showing an attenuated phenotype (Fig. 2K,L). As AB precursors that give rise to ventral and dorsal epidermis express *ceh-16* at earlier stages, we cannot rule out expression of *ceh-16* below detection level in dorsal and ventral epidermis, but we did not detect any defects in the hypodermis of these areas (e.g. the vulvae were perfectly formed in mosaic semi-rescued animals; not shown). We conclude from this analysis that *ceh-16* is required for embryonic seam cell development.

ceh-16/engrailed suppresses fusion of the seam cells by repression of *eff-1* expression

As seam cells that were *ceh-16(–)* and were not rescued in mosaics abnormally fuse to the syncytial hypodermis during embryonic elongation, we hypothesized that *ceh-16* normally inhibits cell fusion and that this inhibition could be executed through the repression of *eff-1*, a gene that is probably necessary for all cell fusion events in *C. elegans* (Mohler et al., 2002; Shemer and Podbilewicz, 2002). In order to test this hypothesis, we constructed animals that were mosaic for *ceh-16* and homozygous for *eff-1(hy21)*. We found that in these animals the cell fusion phenotype of *ceh-16(–)* cells was suppressed (Fig. 3A,B; Table 2). Moreover, in double mutants completely lacking the *ceh-16* rescuing fragment, cell fusion still did not take place as a consequence of *eff-1* absence (Table 2). In addition, *eff-1p::gfp* was ectopically expressed in *ceh-16(–)* seam cells, suggesting that *ceh-16* may be a transcriptional repressor of *eff-1* that is active in the seam cells (Fig. 3C,D; Table 2; see Movie 3 in the supplementary material). When *eff-1* was de-repressed by *ceh-16(RNAi)* in a wild-type background, the seam cells that ectopically express *eff-1* started to fuse (n=7), (Fig. 3E-G). Therefore, these experiments show that *eff-1* is epistatic to *ceh-16*. *eff-1* may be a target of *ceh-16*, and *ceh-16* acts in the seam cells to repress *eff-1* expression. *ceh-16* activity is therefore required to block seam cell fusion with the dorsal and ventral epidermal cells in embryos.

ceh-16/engrailed primes a differentiation cascade in the seam cells

To study whether *ceh-16* is controlling for seam cell fate differentiation, various integrated and non-integrated *gfp* strains expressing early or late seam cell markers were subjected to *ceh-16(RNAi)* (Fire et al., 1998) and their progeny was analyzed (Fig. 4; see Materials and methods). The early markers tested were *elt-5 (=egl-18)*, *nhr-73* and *nhr-74* (Koh and Rothman, 2001; Miyabayashi et al., 1999). The late markers tested were *scm-1*, *wrt-2* and *wrt-5* (not shown) (Aspock et al., 1999; Koh and Rothman, 2001). We found that the progeny of transgenic animals subjected to *ceh-16(RNAi)* showed the typical epidermal defects described in Fig. 2, with the same penetrance for *ceh-16(RNAi)* (Table 1). These defects were accompanied in all cases by diminished or abolished seam cell marker expression, including that of the GATA factor ELT-5(=EGL-18) (integrated strain, n>20; Fig. 4A-B,G-H), known to be necessary for seam cell specification (Koh and Rothman, 2001), and the nuclear hormone receptors NHR-73 (extrachromosomal array, downregulation in 8/8 early embryos [control 2/12]; Fig. 4C-D,I-J) and NHR-74 (extrachromosomal array, downregulation in 9/10 early embryos [control 3/16]; Fig. 4C-D,I-J) (Miyabayashi et al., 1999). The fact that *ceh-16* regulates *nhr-73/74* is interesting, because *elt-5* does not (Koh

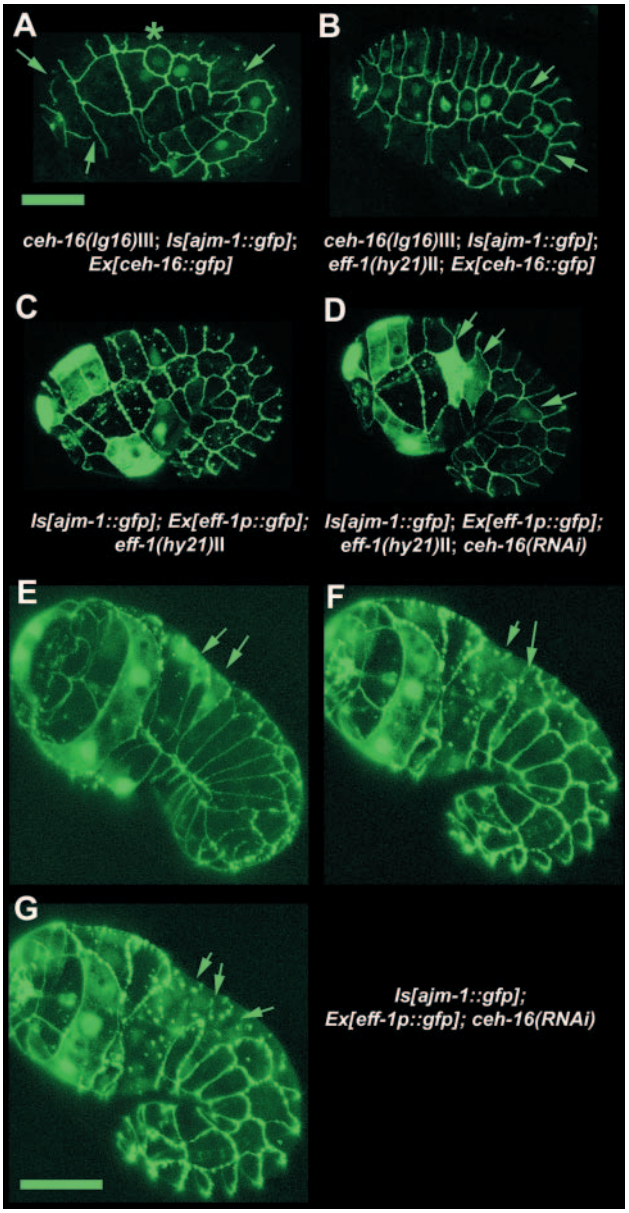

Fig. 3. *eff-1* expression is repressed by *ceh-16* in the embryonic seam cells. (A) *ceh-16(lg16)* mosaic for the rescue marker *ceh-16::gfp* shows seam cell fusions (arrows) and cell migrations (asterisk) (compare Fig. 2). (B) Mosaic animal as in A crossed into *eff-1(hy21)*; suppression of fusion (arrows). (C) *eff-1(hy21)* animal transgenic for an *eff-1::gfp* transcriptional construct that is expressed in cells committed to fuse. (D) as in C, but in addition this animal is *ceh-16(RNAi)*. Ectopic *eff-1::gfp* expression (de-repression) and ectopic migration in the seam cells (arrows) as a result of *ceh-16* inactivation. (E-G) *ceh-16(RNAi)* embryo transgenic for *eff-1::gfp* taken at three time points: *eff-1* expression (*eff-1p::gfp*) in seam cells is de-repressed, resulting in fusion (arrows). All embryos are transgenic for *ajm-1::gfp*. All animals are at 1.5-fold stage except for E (bean stage). Scale bars: 10 μm.

and Rothman, 2001). These results suggest that *ceh-16* is a candidate for an early seam cell 'determinant' required before *elt-5* expression. To strengthen this hypothesis, transgenic animals in which *ceh-16* was expressed ubiquitously under the control of a heatshock promoter were studied (Stringham et al., 1992). L1 larvae expressing *ceh-16* ectopically showed *elt-5::gfp* expression in the dorsal epidermis, where *elt-5* is normally never seen [48% (58/122)]. By contrast, control

Table 2. *ceh-16* represses *eff-1/fusogen*-mediated cell fusion in the seam cells

		Seam cells									
Animal	Description	H0	H1	H2	V1	V2	V3	V4	V5	V6	T
1-13	*ceh-16(–);eff-1(–)*	–	–	–	–	–	–	–	–	–	–
14	*ceh-16* mosaic; *eff-1(–)*	+	–	+	+	+	–	+	–	+	+
15		+	+	+	+	+	–	+	–	+	+
16		+	–	–	–	–	–	–	–	–	–
17		+	+	+	+	+	–	+	–	+	+
18		+	+	+	+	+	–	+	–	+	+
19		+	+	+	+	+	–	–	+	–	–
20		+	+	+	+	+	–	+	–	+	–
21		+	+	+	–	–	–	+	+	–	+
22		+	+	+	–	–	–	–	–	–	–
23	*ceh-16 (RNAi)*; *eff-1(–);* Ex [*eff-1p::gfp*]*	–	–	–	–	–	–	–	–	–	–
24		–	–	–	–	–	–	–	–	–	–
25		–	–	–	–	–	–	–	–	–	–
26		–	–	–	–	–	–	–	–	–	–
27		–	–	–	–	–	–	–	–	–	–

–, no CEH-16::GFP in the nucleus or ceh-16(RNAi); +, CEH-16::GFP in the nucleus.

Genotypes 1-13, double homozygous animals; 14-22, animals that are mosaic for the rescuing *ceh-16::gfp* construct, showing suppression at single cell resolution (see also Fig. 3B); 23-27, animals showing ectopic expression of *eff-1p::gfp* in the seam cells due to mosaic expression of the *eff-1p::gfp* extrachromosomal array.

Grey shading indicates ectopic expression of *eff-1p::gfp*.

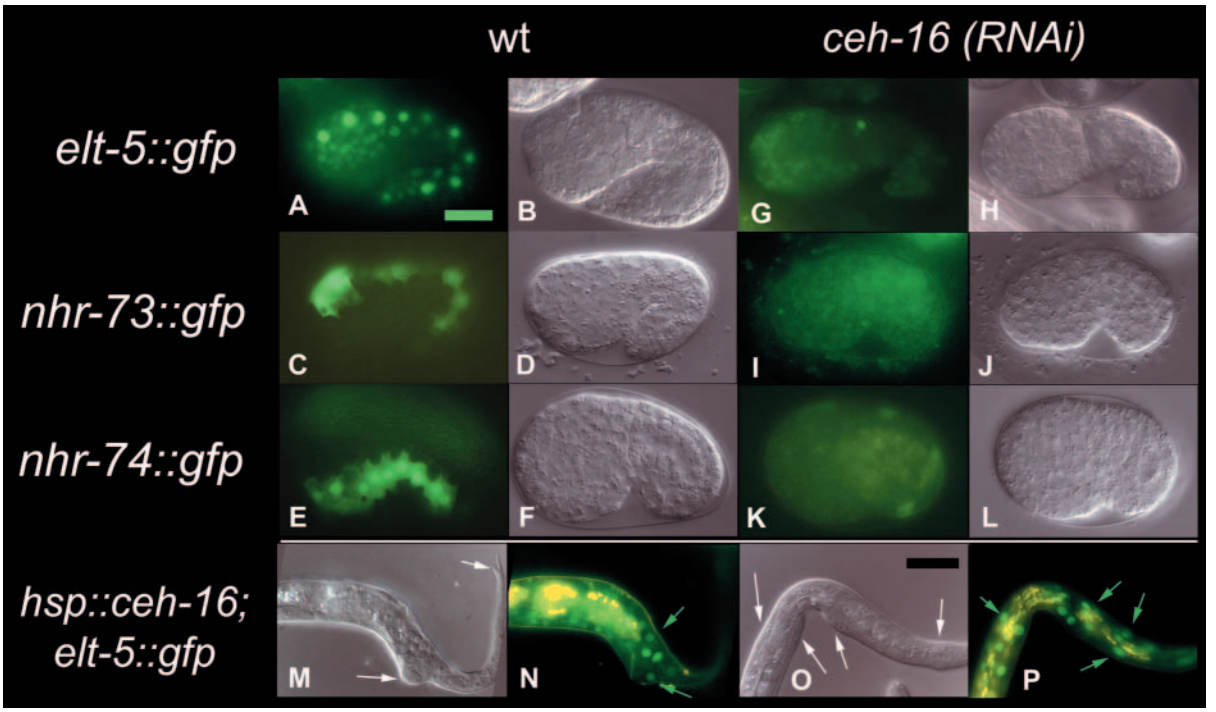

Fig. 4. *ceh-16* regulates early seam cell markers. (A,C,E) Wild-type expression by means of integrated *gfp* constructs [*elt-5* (Koh and Rothman, 2001)] or extrachromosomal arrays [*nhr-73* and *nhr-74* (Miyabayashi et al., 1999)]. (B,D,F) corresponding Nomarski micrographs. (G-L) Corresponding *gfp* strains in which *ceh-16* was knocked down by *ceh-16(RNAi)*. (K,L) Dorsal view. All the markers were downregulated and all animals show the phenotypic hallmarks of *ceh-16(–),* although to a lesser extent due to lower penetrance in RNAi experiments (Table 1). (M-P) *ceh-16* ectopically induces *elt-5::gfp* expression. Ectopic expression of *ceh-16* is achieved by a heatshock-inducible promoter (see Materials and methods). (M) Effects of *ceh-16* misexpression in the epidermis; upper arrow points to irregularities at the tip of the tail, which are reminiscent of failed fusion events [similar as in *eff-1* mutants (Mohler et al., 2002)]. (N) Ectopic expression of *elt-5::gfp* (arrows). (O) Similar to M (upper rightmost arrow points to the anus). (P) Lower arrow (also entire lower margin of the larva) points to ectopic dorsal expression of *elt-5::gfp*. Scale bars: 10 μm for all embryos; 20 μm for all L1 larvae.

animals transgenic for the heatshock vector alone showed no ectopic *gfp* staining (0/138) (Fig. 4M-P). Thus, *ceh-16/engrailed* acts via *elt-5*. This is further confirmed by the fact that in *elt-5(RNAi)* animals seam cells fuse (Koh and Rothman, 2001), and this fusion phenotype is also due to de-repression of *eff-1* (de-repression of *eff-1::gfp* was observed in 4/20 *elt-5(RNAi)* embryos; see Fig. 3 in the supplementary material).

Moreover, analysis of semi-rescued mosaic L1 larvae (>50) revealed that in animals that developed to adult stage individual seam cells that had not expressed *ceh-16* during embryogenesis failed to differentiate into larval seam cells, indicated by the absence of alae formation [specific cuticular structures secreted by the seam cells in L1 stage (Fig. 5A,B)]. Together, these experiments suggest that *ceh-16* is required for early seam cell differentiation/specification.

Differentiation of the seam cells is independent of fusion and requires *ceh-16/engrailed*

As stated above, we have observed regulation of early and late seam cell markers, as well as the loss of the alae morphological structures indicative of the seam cell differentiation. One could argue that *ceh-16(–)* seam cells during embryogenesis ectopically fuse with the hypodermal syncytium, and therefore, as part of the syncytium, lose their ability to express late markers or to form alae. The loss of differentiation might, therefore, be due to indirect effects and not due to the lack of the specific onset of differentiating genes regulated by *ceh-16*. To exclude this possibility, we tested *ceh-16* mutants in which fusion did not take place. This was accomplished in one of two ways: First, *ceh-16(–)* seam cells failed to secrete alae also when they 'occasionally' escaped fusion (Fig. 5C,D). Second, in the fusion-incompetent background of *eff-1(hy21)*, 25/25 animals showed alae-gaps in *eff-1(–);ceh-16(–)* mosaic L1 larvae (not shown). This experiment strongly suggests that *ceh-16* is required for the determination of seam cell fate and not only to repress cell fusion.

ceh-16/engrailed is required to maintain correct seam cell positioning during embryogenesis

We had observed that *ceh-16(–)* seam cells migrate (Fig. 2). We decided to analyze this phenotype in more detail. Of the mosaic embryos (n=20), 70% displayed abnormal cell positioning phenotype (bean to comma stage), with no directional preference (see Fig. 2E,F; arrows). Do *ceh-16(–)* cells in mosaic animals leave their position (migration), or is the loss of collinear arrangement due to earlier events, as *ceh-16* is expressed earlier (Fig. 1C,E)?

To answer this question, *ceh-16* mosaic embryos in an *eff-1(–)* background were analyzed. In these animals ectopic fusions were not present (Fig. 3B; Table 2). As in a *ceh-16(–);eff-1(+)* background, *ceh-16(–);eff-1(–)* cells were not in their normal position. Of 40 embryos tested from bean to 1.5-fold stage of elongation, 33 embryos showed a seam cell-defective phenotype (83%). Out of 62 aberrant seam cells, 48 (77%) had either a minor or strong projection intercalating with the ventral P cells. The minor projection often preceded a more pronounced migration of the entire cell, visible when the embryo was re-analyzed at a later stage (1.8-fold). The remaining 23% of the *ceh-16(–)* seam cells displayed migration toward the dorsal side (when animals were analyzed at a later stage). Unlike in *eff-1(–)* and wild-type animals, in *eff-1(–);ceh-16(–)* double mutants the shape of the seam cells was not wild type and the cell belt margins were no longer straight (see Movie 2 in the supplementary material), but were dented or intercalated (Fig. 6; see Movie 3 in the supplementary material). Moreover, migration can be observed live in a time-lapse experiment, where a *ceh-16(–)* seam cell, marked by the ectopic expression of *eff-1p::gfp*, migrated dorsally (see Movie 3 in the supplementary material: in the depicted experiment, not all the seam cells expressed GFP, probably due to mosaic expression of the extrachromosomal array). We conclude from these experiments that *ceh-16* is required for the maintenance of correct boundaries between the lateral rows of seam cells and the ventral and dorsal row of epidermal cells during embryonic development.

Discussion

In this work we have cloned and characterized the cDNA of

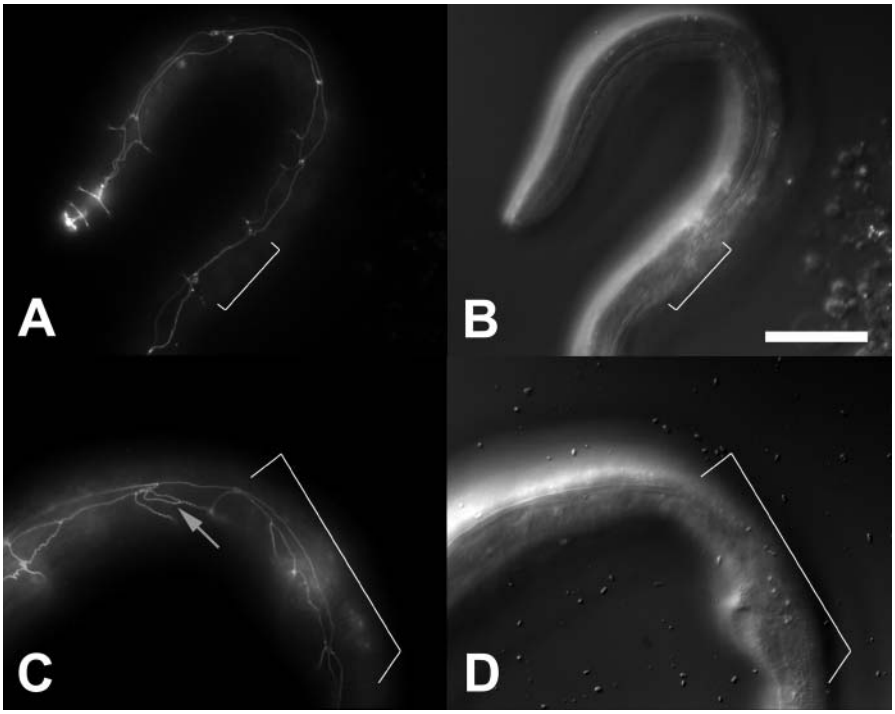

Fig. 5. *ceh-16* is required for the differentiation of seam cells. (A) fused seam cell in a *ceh-16* mosaic animal (bracket; see also Fig. 1). (B) Nomarski micrograph of the same animal, showing the lack of alae formation at the position where the seam cell fuse (bracket). (C) Seam cell that neither fused nor expressed *ceh-16::gfp* during embryogenesis (bracket); arrow shows a seam cell that moved ventrally and is not elongated. (D) The seam cell did not express *ceh-16::gfp* embryonically or generated alae (bracket). Both animals are L1. Scale bar: 20 μm.

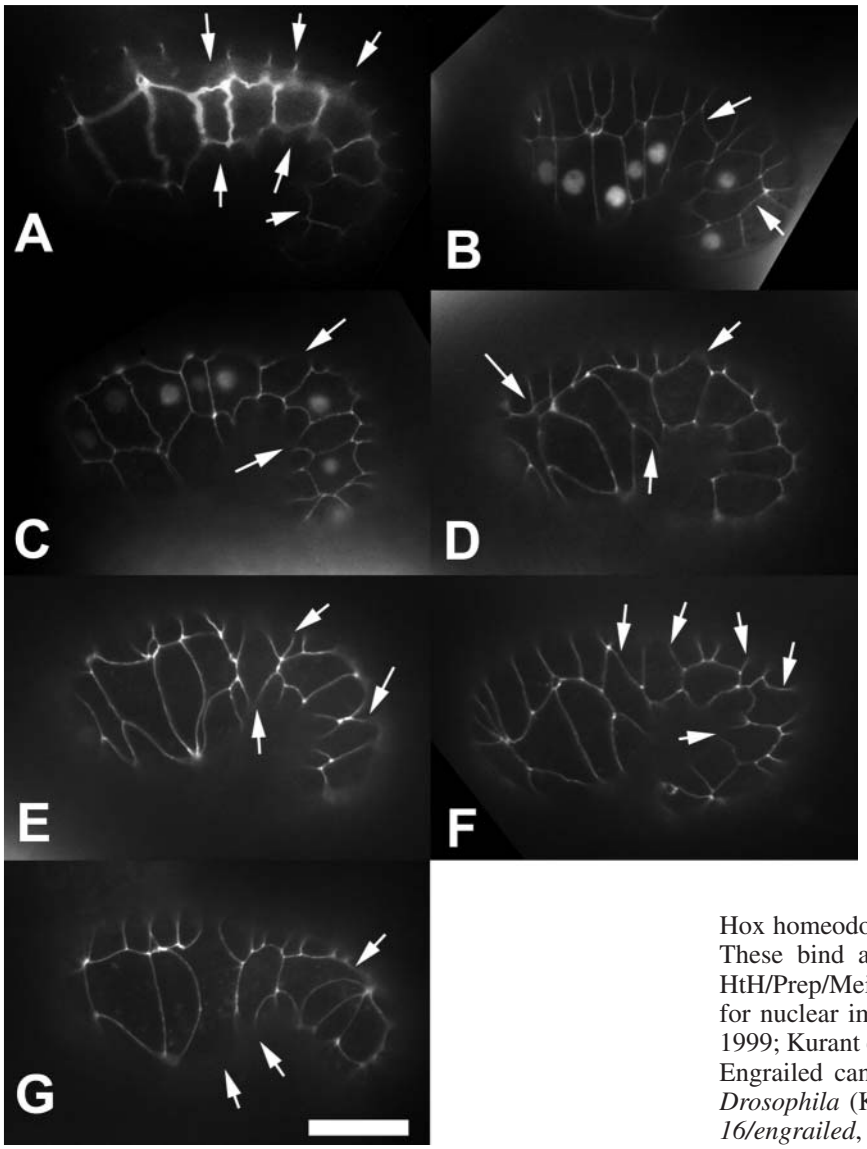

Fig. 6. *ceh-16* is required for correct segregation of the seam cells into a straight row of cells. (A) Wild-type animal with straight compartment delineations (arrows). (B,C) Seam cells in mosaic animals (see also Fig. 1) that lack *ceh-16::gfp* expression (arrows) migrate and/or lose their shape. (D-G) Examples of mosaic embryos that did not show any *ceh-16::gfp expression*. Less penetrant phenotype is shown in C; more severe seam cell movements are shown in D-G (arrows point at extreme situations). Scale bar: 10 μm.

ceh-16, the only *engrailed-like* gene in *C. elegans*. In *Drosophila* and vertebrates *engrailed-like* genes have been duplicated during evolution (Gibert, 2002), whereas in the nematode *Caenorhabditis elegans* only one *engrailed-like* gene exists (this work). Phenotypic analysis of mutants and mosaic animals revealed that *ceh-16/engrailed* is required embryonically for the differentiation of the seam cells. The animals lacking *ceh-16/engrailed* activity died during embryogenesis, most probably due to defective elongation. Moreover, seam cells that failed to express *ceh-16/engrailed* fused to surrounding *ceh-16/engrailed (–)* cells in an *eff-1*-dependent manner. We also showed that *eff-1* is epistatic to *ceh-16/engrailed* and that *ceh-16/engrailed* acts as a transcriptional repressor for *eff-1* in the seam cells. In addition, the function of *ceh-16/engrailed* is required to maintain the embryonic seam cell fate and the linear lateral position of the seam cells.

ceh-16/engrailed the only ortholog of the *engrailed* genes in the nematode *C. elegans*

CEH-16 shares the archetypal structure of *engrailed* class proteins from arthropods, annelids, chordates and vertebrates (see Fig. 1 in the supplementary material). Besides the homeodomain referred to as Engrailed Homology domain 4 (EH4), CEH-16 possesses at least EH1, EH2 and EH3 [of the five known EH domains (Gibert, 2002)]. EH1 is constituted by the *engrailed* repressor domain, which binds to Groucho in *Drosophila*. Groucho can act as a co-repressor of transcription (Jimenez et al., 1997), is conserved in *C. elegans* and binds to the EH1 domain, which is also present in *unc-4* (Winnier et al., 1999). Interestingly, we have shown that *ceh-16/engrailed* represses the transcription of *eff-1*, but we do not know whether this repression is direct or indirect, nor if it is dependent on Groucho-like genes. EH2 and EH3 are involved in the binding of Hox homeodomain co-factors such as Pbx/Exd in *Drosophila*. These bind again to other homeodomain co-factors of the HtH/Prep/Meis gene family; these interactions are important for nuclear import (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Kurant et al., 1998; Rieckhof et al., 1997). Exd, HtH and Engrailed can form a functional triple repressor complex in *Drosophila* (Kobayashi et al., 2003). In *C. elegans*, like *ceh-16/engrailed*, the orthologs of Exd (=*ceh-20/40*) and Hth (=*unc-62*) are also involved in embryonic epidermal development (Van Auken et al., 2002). As the EH2/3 domains in CEH-16 are present, CEH-20/40 and UNC-62 might also be co-factors of CEH-16/Engrailed in *C. elegans*.

Cell fusion and differentiation – two separable functions controlled by *ceh-16/engrailed*

Cell fusion has been shown to control cell fates. When cell fusion is blocked during postembryonic development in *eff-1* mutants, unfused VPCs can also respond to neighboring signals and adopt vulval fates (reviewed by Shemer and Podbilewicz, 2003). The result is an ectopic and non-functional vulva (Mohler et al., 2002).

We have shown that the regulation of epidermal cell fusion is also crucial during embryonic development. Seam cells act as a non-fusing 'inter-zone' between the dorsal and the ventral areas of the embryo. Although fusion repression prevents the seam cells from becoming a part of the dorsal syncytium, we have shown by bypassing ectopic fusions in an *eff-1* mutant background that *ceh-16/engrailed* is necessary for the differentiation of the seam cells, also in a fusion negative genetic background. In the seam cells that lack *ceh-*

16/engrailed, *eff-1* is de-repressed. The de-repression of *eff-1* occurs simultaneously with expression of *eff-1* in the other epidermal cells (e.g. in the forming hyp7 syncytium). *ceh-16/engrailed* therefore installs an additional program in a subset of cells otherwise committed to behaving like the surrounding epidermal cells. In summary, *ceh-16/engrailed*, during embryonic development, primes a transcriptional cascade necessary for seam cell differentiation. To allow this separate seam cell differentiation, and to maintain the lateral epidermal cell fate, *ceh-16/engrailed* also represses the fusion of the seam cells.

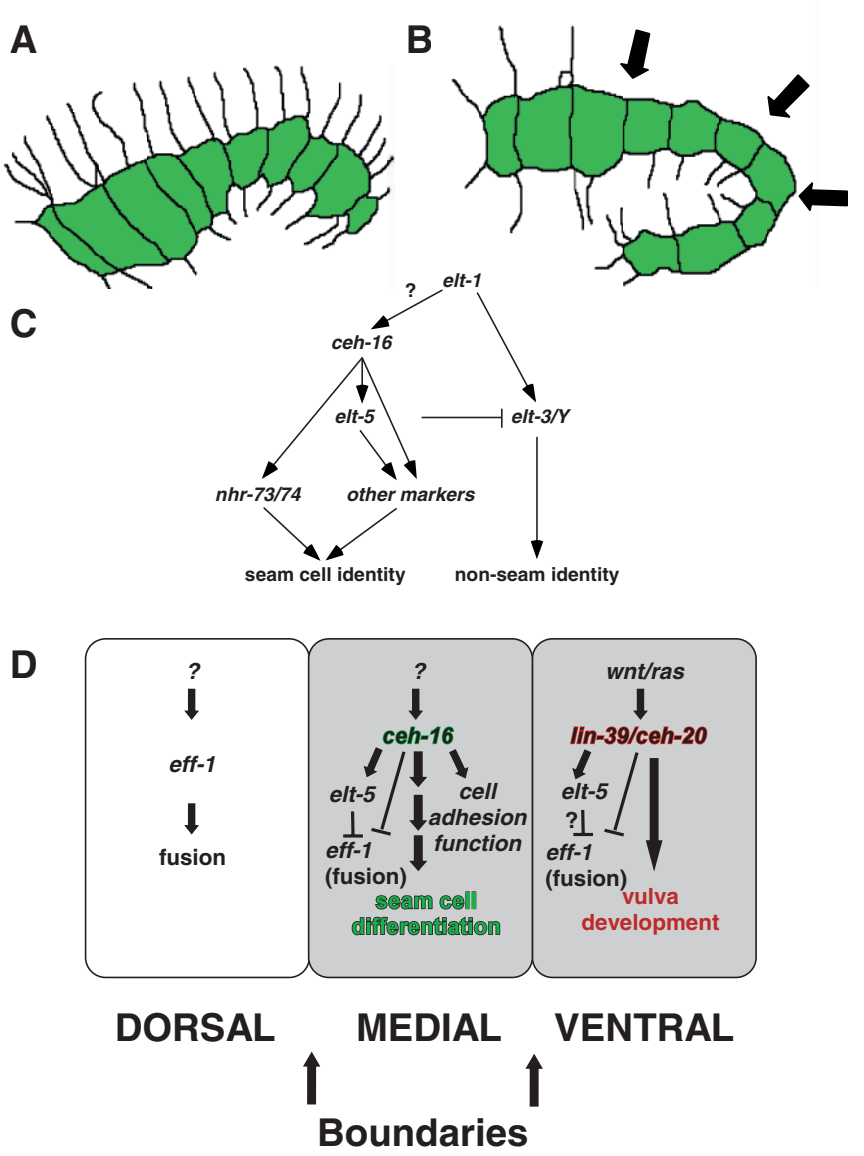

Fig. 7. Model for the function of *ceh-16/engrailed* in *C. elegans*. (A) *ceh-16/engrailed* keeps the seam cells in a linear organization (green), due to its repression of seam cell fusion and cell migrations. (B) During elongation, hyp7 (dorsal) start to fuse; *ceh-16/engrailed* acts as a fusion repressor and as a regulator of seam-cell differentiation in order to maintain a straight boundary. At this stage genes important for 'seam-cell-fate' are turned on/regulated by *ceh-16/engrailed*. (C) Transcriptional cascade regulating seam cell fate. (D) Summary of genetic regulatory cascades in the three rows of epidermal cells preceding and during the 1.5-fold stage: Dorsal: *eff-1* is expressed and required for the fusion of hyp7 into a syncytium. It is not known which genes regulate *eff-1* expression in the dorsal epidermis. Medial or lateral: in the seam cells *ceh-16/engrailed* represses the expression of *eff-1* (directly or indirectly). This is necessary to allow *ceh-16/engrailed* to activate genes required for correct positioning of the seam cells (cell adhesion molecules) and for the differentiation of the seam cells (*elt-5* etc.). Genes that regulate *ceh-16/engrailed* in *C. elegans* are unknown. Ventral: analogous to *ceh-16/engrailed* in the seam cells, *lin-39*/HoxD4/Dfd and *ceh-20*/Exd are required for the regulation of *elt-5* and for the repression of *eff-1* to allow vulva formation (Koh et al., 2002; Shemer et al., 2004). During postembryonic development, the seam cells act as a belt with two straight boundaries that are lost in the absence of *ceh-16/engrailed*.

ceh-16/engrailed commits the lateral epidermis to seam cell fate in part by regulating the expression of *elt-5* (=*egl-18*). As mutations of *elt*-5, like *ceh-16*, have also been shown to prevent cell fusions and, to a minor extent, inappropriate cell migration of the seam cells (Koh and Rothman, 2001), we suggest a regulatory cascade in which *ceh-16* controls *elt-5*, which may repress *eff-1* expression and participates in anti-migratory mechanisms (Fig. 7C,D). Although we are able to ectopically express *elt-5* by misexpression of *ceh-16* we do not know if *elt-5* is a direct target of *ceh-16*. Moreover, supporting an indirect regulation, we found no putative *ceh-16/engrailed* binding site in the *elt-5* locus by screening *in silico*, using the reported *Drosophila engrailed* binding sequence (Solano et al., 2003). Are all the functions of *ceh-16/engrailed* mediated by *elt-5*? Although the phenotype of *elt-5* larvae is very similar to the one seen in *ceh-16* mutants, we think that this is not the case. Koh et al. (Koh et al., 2002) showed that *elt-5* controls many markers of the seam cells. But *nhr-73* and *nhr-74* are not regulated by *elt-5*, so the authors speculated that there must be an additional factor X, which might act in parallel to *elt-5*. We have shown that *ceh-16* regulates *nhr-73/74* and *elt-5*. So *ceh-16* may be the factor X, which is placed upstream of *elt-5* (Fig. 7C). Interestingly, in the ventral region of the epidermis, cell fusions are controlled by the expression of another homeobox repressor, *lin-39/HOXD4/Dfd* and *ceh-20/Exd* (Shemer and Podbilewicz, 2002), which also act through *elt-5* (Koh et al., 2002). In this region, *elt-5*, controlled by *lin-39*, is essential for vulva formation. Therefore, it seems that a concerted spatial-temporal (lateral-ventral) expression of different homeodomain proteins controls differentiation of respective epidermal structures by recruiting in part the same factors (such as *elt-5* and *eff-1*; Fig. 7D). The occurrence of *elt-5* in lateral and ventral domains of the epidermal cells might be required for the regulation in both areas of *eff-1* (Fig. 7D).

The expression of *lin-39* in VPCs is regulated via *wnt/ras* signaling pathways (Eisenmann et al., 1998). But how is the expression of *ceh-16/engrailed* regulated? The exclusive epidermal expression of *ceh-*

16/engrailed in the seam cells cannot be explained by lineage relationships as it is for *engrailed*-positive cells within a compartment in *Drosophila* (reviewed by Dahmann and Basler, 1999). One possible way to explain such a simultaneous expression of the same gene in the same topological area is the existence of an extracellular signal directing the expression of *ceh-16/engrailed* from an organizing zone. There is so far only one extracellular signal known to control embryonic epidermal development in *C. elegans*: the ephrin signaling pathway (Chin-Sang et al., 1999; Chin-Sang et al., 2002; George et al., 1998). The ephrins act in the neuroblasts, regulating the ventral closure of the overlying epidermal cells. This function is indirect and the signaling pathway mediating this function is unknown. We consider it unlikely that *ceh-16/engrailed* expression is dependent on ephrin function, as the phenotype we have observed occurs in animals that have already performed ventral closure. It is, however, conceivable that ephrin signaling attracts *ceh-16(–)* seam cells to the ventral side at later stages (see below).

ceh-16/engrailed blocks ectopic cell migrations

The mosaic analyses and the time-lapse recordings show that the seam cells form a straight cell line that acts as a migration barrier (Fig. 7A,B). Differential cell adhesion properties may account for cells segregating from this line, as in mosaic animals *ceh-16(–)* cells invade the neighboring tissues. At this stage we cannot say whether this phenomenon is cell-autonomous or not. Since *ceh-16/engrailed* is expressed earlier in precursors of ventral and dorsal epidermis, it may be necessary for correct migration events there as well but not detectable by our experimental means. That *ceh-16/engrailed* may elicit such a phenotype might be due to the de-regulation of homophilic cell-surface molecules regulating adhesion and/or cell motility. Analogously, in *Drosophila* such mechanisms have been hypothesized to be involved in the formation/maintenance of compartment boundaries, where the additional paracrine function of *hh* is required for the boundary to be held in place (Dahmann and Basler, 1999). Studies based on rRNA have demonstrated that nematodes had previously been misplaced, and their true position is in a sister group of the arthropods (Adoutte et al., 2000; Aguinaldo et al., 1997). Therefore their evolutionary relationship to arthropods is closer than expected. The question arises whether *engrailed* in nematodes is controlling cell migration mechanisms by regulating the same molecules as in arthropods (*Drosophila*). To answer this question the identification in both species of these cell-adhesion molecules (controlled by *engrailed*) is required.

In conclusion, this work shows how *engrailed* patterns the embryonic epidermis of *C. elegans*. For this purpose, *ceh-16/engrailed* acts as a differentiation factor, as a cell migration inhibitor, and we describe for the first time how an *engrailed-like* gene controls animal developmental processes also by the regulation of cell fusion.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/4/739/DC1

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