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The *Pristionchus* HOX gene *Ppa-lin-39* inhibits programmed cell death to specify the vulva equivalence group and is not required during vulval induction

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SUMMARY

In the two nematode species *Caenorhabditis elegans* and *Pristionchus pacificus* the vulva equivalence group in the central body region is specified by the Hox gene *lin-39*. *C. elegans lin-39* mutants are vulvaless and the vulval precursor cells fuse with the surrounding hypodermis, whereas in *P. pacificus lin-39* mutants the vulval precursor cells die by apoptosis. Mechanistically, LIN-39 might inhibit non-vulval fate (cell fusion in *C. elegans*, apoptosis in *P. pacificus*), promote vulval fate or do both. To study the mechanism of *lin-39* function, we isolated *P. pacificus* cell death mutants and identified mutations in *ced-3*. Surprisingly, *P. pacificus ced-3; lin-39* double mutants form a functional vulva in the absence of LIN-39 activity. Thus, in *P. pacificus lin-39* specifies the vulva equivalence group by inhibiting programmed cell death. Furthermore, these data reveal an important difference in a later function of *lin-39* between the two species. In *C. elegans*, LIN-39 specifies vulval cell fates in response to inductive RAS signaling, and in *P. pacificus* LIN-39 is not required for vulval induction. Thus, the comparative analysis indicates that *lin-39* has distinct functions in both species although the gene is acting in a homologous developmental system.

Key words: Apoptosis, *Caenorhabditis elegans*, Homeotic genes, *Pristionchus pacificus*, Vulva, *Ppa-lin-39*

INTRODUCTION

In organisms as diverse as vertebrates, insects or nematodes a group of highly conserved *Antennapedia*-class homeobox genes, called the HOX or HOM-C genes, is involved in patterning the anteroposterior (A/P) axis (reviewed by Krumlauf, 1994; Lawrence and Morata, 1994; Salser and Kenyon, 1994). HOX genes are expressed in restricted domains and are thought to act as transcriptional regulators to provide regional information and cell-type specificity. Although the temporal and spatial distribution of Hox transcripts is crucial for the establishment of the distinctive morphological structures in animals, we have a limited understanding of how Hox genes function in the specification of cell fates and how the functional specificity of these genes changes during evolution. We have taken advantage of the invariability of the nematode cell lineage to study Hox gene function and evolution during vulva development in the nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*.

In *C. elegans*, four HOX genes have been identified (reviewed by Salser and Kenyon, 1994). The genes *lin-39*, *ceh-13*, *mab-5* and *egl-5* resemble the *Drosophila* genes *Deformed/Sex-combs-reduced*, *labial*, *Antennapedia/Ultrabithorax/abdominal-A*, and *abdominal-B* respectively. Mutations in two of these genes, *lin-39* and *mab-5*, affect the fate specification of precursor cells in the mid-body region that give rise to the hermaphrodite vulva (Wang et al., 1993; Clark et al., 1993; Clandinin et al., 1997; Maloof and Kenyon, 1998). Thereby, the gene *lin-39* plays a central role as it is used twice during vulval cell fate specification. Early in the first larval stage (L1), *lin-39* functions as a homeotic gene that specifies the vulva equivalence group. *lin-39* mutant animals are vulvaless and the vulval precursor cells (VPCs) express fates of more anterior or posterior homologs which undergo cell fusion with the hypodermal syncytium *hyp7* (Wang et al., 1993; Clark et al., 1993). In the third larval stage (L3), *lin-39* directs vulva cell fates as a transcription factor that acts in response to inductive EGF/RAS/MAPK signaling (Clandinin et al., 1997; Maloof and Kenyon, 1998).

A theme central to the genetic analysis of development is, whether the role of a gene product is permissive or instructive in cell fate specification. Whereas the function of LIN-39 during vulval induction is clearly instructive, little is known about the earlier homeotic role of LIN-39. The cell fusion phenotype of the VPCs in *C. elegans lin-39* mutations does not allow insight into the molecular mechanism of LIN-39 function. Mechanistically, LIN-39 activity might repress cell fusion of the VPCs, promote vulval fate, or do both. This issue has not been addressed so far, as no cell fusion mutants have been isolated in *C. elegans*. Such mutants would allow one to

study *lin-39* function genetically by generating double mutants between *lin-39* and a cell fusion defective mutant. For example, if the double mutant did form a vulva then LIN-39 is not instructive and acts to block cell fusion in the wild-type animal.

We have studied the function and evolution of *lin-39* in the nematode *P. pacificus*, which has been described as a 'satellite' organism for a functional comparative approach, because genetic, molecular and cell-biological tools can be used in a way similar to the genetic model organism *C. elegans* (Sommer and Sternberg, 1996; Sommer et al., 1996; Eizinger and Sommer, 1997; Jungblut and Sommer, 1998). In *P. pacificus*, the *lin-39* homolog *Ppa-lin-39* specifies the central body region (Eizinger and Sommer, 1997). *Ppa-lin-39* mutants are vulvaless, but the VPCs undergo apoptosis in *P. pacificus* instead of cell fusion (Eizinger and Sommer, 1997). As in *C. elegans*, *P. pacificus* LIN-39 might have a permissive or instructive role during the specification of the vulva equivalence group.

Here, we report the isolation of cell death defective mutations in *P. pacificus*. Molecular characterization has shown that this phenotype is caused by point mutations in the *P. pacificus* homolog of *ced-3*, which has been identified as a key regulator during programmed cell death in *C. elegans*. We have used such mutations to further study the mechanism of *Ppa-lin-39* function by creating *Ppa-lin-39; Ppa-ced-3* double mutants. Such double mutants have a functional vulva in the absence of LIN-39 activity, indicating that *P. pacificus* LIN-39 has a permissive function by inhibiting programmed cell death. Furthermore, these data reveal a surprising difference in the later function of *lin-39*. Unlike in *C. elegans*, where LIN-39 specifies vulval cell fate in response to RAS signaling, *Ppa-lin-39* is dispensable for later vulval cell fate specification.

MATERIALS AND METHODS

Strains and cultures

P. pacificus var. California was the wild-type parent of all strains used in this work. Strains were handled and maintained as described by Sommer et al. (1996). Animals were grown at 20°C and Nomarski observation was done according to standard protocols (Sommer and Sternberg, 1996). For general worm culture see also Epstein and Shakes (1995).

EMS mutagenesis

Mixed stage animals were washed off the plates in M9 buffer, and ethyl methanesulphonate (EMS) added, to a final concentration of 50 mM, for 4 hours at 20°C. The suspension was washed in M9 five times and the worms were spotted onto the surface of two NG plates. After 1 h excess liquid had been absorbed and individual motile L4 hermaphrodites were picked individually to plates. In the Nomarski screen, F_2 progeny of the mutagenized animals were picked individually to plates and their progeny were analyzed under Nomarski optics. Usually, 10 F_3 animals were analyzed per F_2 parent. Altogether, 3500 clonal F_2 lines were analyzed that way and three original cell death defective mutants were isolated.

In the suppressor screen, *Ppa-lin-39(tu29)* mutant animals were mutagenized and motile L4 hermaphrodites were picked individually to plates. As these animals are vulvaless, eggs are seen only rarely on plates. All progeny generated by self-fertilization hatch inside their mothers body and are released when the mother bursts. Plates were incubated at 20°C for three to four generations and eggs were transferred to a new plate. If a suppressor mutation was present that converted the egg-laying defective phenotype to an egg-laying positive phenotype, the number of eggs per plate increased. Young adult animals of such plates were analyzed for the presence of a vulva and additional Pn.p cells using Nomarski microscopy. Mutant hermaphrodites were backcrossed (Nomarski screen) or outcrossed (suppressor screen) using wild-type males. All mutants were backcrossed several times. Complementation tests were carried out using homozygous males of the suppressor strains and crossed them to *Ppa-ced-3(tu79)* hermaphrodites that were marked with the recessive visible mutation *Ppa-unc-1* (Sommer et al., 1996). Cross progeny from such matings are wild-type for the visible marker (being *Ppa-unc-1*/+) and cell death defective indicating that the suppressors are alleles of *Ppa-ced-3*.

General DNA analysis

General DNA manipulation and analysis were done using standard protocols described by Sambrook et al. (1989). The genomic and the cDNA library screened to obtain *Ppa-ced-3* clones has been described by Sommer et al. (1996). Subclones were made using the pBluescript vector (Stratagene Cloning Systems, La Jolla, CA) and the M13 derivative mtvh (Schlötterer and Wolff, 1996).

PCR experiments and mutant sequencing

A PCR fragment of *Ppa-ced-3* encoding a region of 31 amino acids, was used for screening a genomic and a cDNA library respectively. PCR was performed using the following degenerate primers: RS118, 5′-CTGGTCATYMTRWCNCAYGG-3′ and RS127, 5′-CTGGAC-RAAGACRATYTTNGGYTT-3′. To isolate the amino-terminal portion of *Ppa-ced-3*, RT-PCR was performed. *P. pacificus* poly(A)+ RNA was reverse transcribed using M-MuLV-Reverese Transcriptase (Stratagene Cloning Systems, La Jolla, CA) and the primer RS202, 5′-CAGTCTCTTCATATCCAAT-3′. In this reaction, approximately 150 ng poly(A)+ RNA were used in a 20 μl volume. 1 μl of this reaction was used in a standard PCR reaction using the SL-1 specific primer RS 234, 5′-GGTTTAATTACCCAAGTTTGAG-3′ and the primer RS 202 that had been used for the reverse transcription. 1 μl of PCR product was used for a nested PCR using the primer RS 234 and the primer RS 203 5′-AGAATACAGACAATGGCAGA-3′ in the carboxy-terminal portion of the gene. The PCR product was cloned into M13mtvh and sequenced.

For mutant sequence analysis, DNA was isolated from two independent batches of animals, amplified by three independent PCR reactions, cloned into M13mtvh, and sequenced. We analyzed the complete coding region and at least 10 base pairs on either side of splice sites in 6 alleles and found point mutations in all of them.

Generation of double mutants

The *Ppa-lin-39* alleles *tu2* and *tu29* differ with respect to the extent of cell survival of the potential VPCs (Eizinger and Sommer, 1997). The allele *Ppa-lin-39(tu2)* represents a strong allele, where on average only 0.11 VPCs survive per animal. The allele *Ppa-lin-39(tu29)* has a weaker phenotype, and 0.81 VPCs survive per animal. Both alleles were marked with the linked recessive visible mutation *Ppa-dpy-1(sy304)*. *Ppa-lin-39(tu2) Ppa-dpy-1(sy304)* double mutant hermaphrodites were crossed with *Ppa-ced-3(tu79)* or *Ppa-ced-3(tu84)* homozygous males generating triple heterozygous hermaphrodites as cross progeny. In the F_2 generation, segregants that are *dumpy* and egg-laying defective were picked, two third of which should be heterozygous for *Ppa-ced-3*. Of such plates, we observed one-quarter of the F_3 segregants to be triple mutant. *Ppa-dpy-1(sy304) Ppa-lin-39(tu2); Ppa-ced-3(tu79)* and *Ppa-dpy-1(sy304) Ppa-lin-39(tu2); Ppa-ced-3(tu84)* triple mutants have been isolated based on the visible marker phenotype and the presence of 12 Pn.p cells as indicated by Nomarski observation. For simplicity, in the text we refer to *Ppa-dpy-1(sy304) Ppa-lin-39(tu2); Ppa-ced-3(tu79)* triple mutant animals as *Ppa-lin-39(tu2); Ppa-ced-3(tu79)* double mutants. To segregate the mutant chromosomes, such triple mutant

hermaphrodites were crossed with wild-type males and the mutant phenotypes Dumpy, egg-laying defective and cell death defective respectively were reisolated.

RESULTS

Vulva development

The *C. elegans* vulva is a derivative of the ventral epidermis which consists of 12 precursor cells called P1.p through P12.p (Sulston and Horvitz, 1977) (Fig. 1A). These cells adopt either a nonvulval or vulval fate in a position-specific manner (Fig. 1B). During the L1 stage, P(1,2,9-11).p are committed to a nonvulval fate and fuse with the hypodermal syncytium *hyp7*. In contrast, the mid-body cells P(3-8).p remain unfused and form the vulva equivalence group. These mid-body cells are called VPCs, as all cells have the potential to form part of the vulva later in development. The initial decision to become a VPC requires the HOX gene *lin-39* (Wang et al., 1993; Clark et al., 1993): in *lin-39* mutants, P(3-8).p fuse with *hyp7*, like their anterior and posterior lineage homologs and no vulva is formed (Fig. 1B).

LIN-39 activity is also required during *C. elegans* vulva induction in the third larval stage (L3) (Clandinin et al., 1997; Maloof and Kenyon, 1998). In wild-type animals the vulva is formed by the progeny of P(5-7).p (Sulston and Horvitz, 1977) (Figs 1B, 2). Vulval differentiation of P(5-7).p is induced by a signal from the gonadal anchor cell (AC) (Kimble, 1981) that activates a RAS signaling pathway (reviewed in Kornfeld, 1997) (Fig. 2). Using a *hs-lin-39* construct to bypass the early defect, it has been shown that *lin-39* activity is absolutely

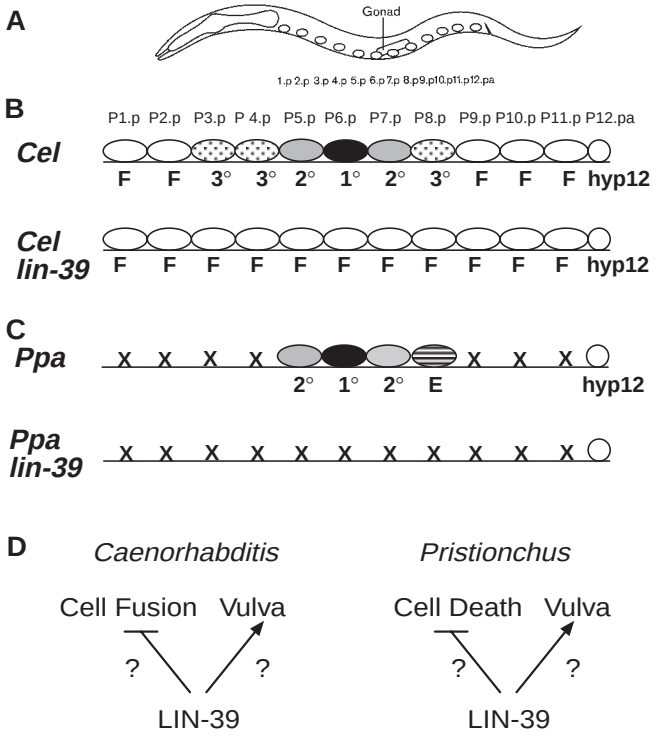

Fig. 1. Schematic summary of ventral epidermal cell fate decisions in *C. elegans* (Cel) (B) and *P. pacificus* (Ppa) (C) in wild-type and *lin-39* mutant animals. F, cell fusion; X, programmed cell death. (A) In the L1 stage in *C. elegans*, the 12 ventral epidermal cells P(1-12).p are equally distributed in the region between the pharynx and the rectum. (B) In wild-type animals P(1,2,9-11).p in the anterior and posterior region fuse with the surrounding hypodermis (white ovals), whereas the VPCs P(3-8).p remain unfused. Later in development they adopt one of three alternative fates as indicated by different symbols (Fig. 2 for details). In *Cel-lin-39* mutants, P(3-8).p undergo cell fusion like their anterior and posterior neighbors. (C) In *P. pacificus*, P(1-4,9-11).p die of programmed cell death, P(5-8).p in the central body region survive. P8.p remains epidermal and the progeny of P(5-7).p form the vulva. In *Ppa-lin-39* mutants, P(5-8).p die of programmed cell death like their anterior and posterior neighbors. (D) Potential mechanisms of LIN-39 function. LIN-39 might repress the non-vulval fates (fusion in *C. elegans* and cell death in *P. pacificus*), promote vulval fate or do both.

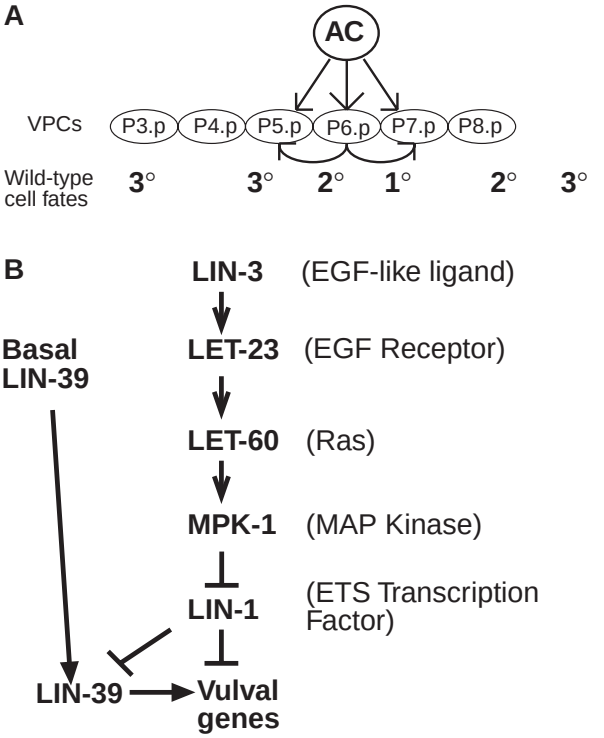

Fig. 2. Schematic summary of vulva induction in *C. elegans*. (A) Cell signaling events controlling VPCs fate decisions. The graded AC signal (arrows between AC and VPCs), along with lateral signaling among the VPCs (arrows pointing from P6.p to P(5,7).p) ensure the invariant pattern 3°-3°-2°-1°-2°-3°. Cells with a 1° and 2° fate generate 8 and 7 cells respectively, all of which participate in the formation of the vulva. 3° cells remain epidermal. (B) A model for vulva induction. Vulval induction is mediated by a conserved EGF/Ras/MAPK signaling pathway. *lin-3* encodes the EGF-like ligand which is expressed in the AC and signals the underlying VPCs (Hill and Sternberg, 1992); *let-23* encodes an EGF-receptor tyrosine kinase (Aroian et al., 1990); *let-60* encodes a Ras molecule acting as a molecular switch (Han and Sternberg, 1990; Beitel et al., 1990), *mpk-1/sur-1* encodes a MAP kinase (Lackner et al., 1994; Wu and Han, 1994) and *lin-1* encodes an ETS-domain transcription factor which functions to inhibit vulva formation (Beitel et al., 1995). In P(5-7).p, AC signaling inactivates *lin-1* which then leads to vulva formation. *lin-1* represses a number of 'vulval genes' including *lin-39* (Maloof and Kenyon, 1998). A basal level of *lin-39* expression is required for the increase of *lin-39* expression in response to Ras signaling (Maloof and Kenyon, 1998).

required in order for RAS signaling to induce vulva formation (Maloof and Kenyon, 1998). If no LIN-39 activity is present during RAS signaling, no vulva is formed. Thus, *lin-39* is required twice during vulva formation. First, LIN-39 specifies the vulva equivalence group in the L1 stage and second, it directs vulva cell fates in the L3 stage in response to RAS signaling.

In *P. pacificus*, the nonvulval cells P(1-4,9-11).p undergo programmed cell death instead of cell fusion (Figs 1C, 6A) (Sommer and Sternberg, 1996). *Ppa-lin-39* specifies the central body region and *Ppa-lin-39* mutants are vulvaless because P(5-8).p undergo apoptosis (Eizinger and Sommer, 1997) (Figs 1C, 6B). Taken together, the *lin-39* mutant phenotype in both species does not distinguish between a permissive or instructive role of LIN-39 (Fig. 1D). To address this question, we decided to further study *lin-39* in *P. pacificus* because programmed cell death is genetically accessible, whereas cell fusion, the nonvulval fate in *C. elegans*, has not been studied using genetic tools. To determine the mechanism of LIN-39 function we isolated cell death defective mutations in *P. pacificus* to generate double mutants between *Ppa-lin-39* and a cell death defective mutation.

Isolation of *Pristionchus* cell death mutants

Cells dying by programmed cell death in *P. pacificus* have similar morphological characteristics as in *C. elegans* and result in visible cell corpses. As in *C. elegans*, the embryonic pharynx is a region of high cell death activity. However, the presence of seven programmed cell death events in the ventral epidermis of *P. pacificus* hermaphrodites makes this region the most convenient for analysis of the presence or absence of programmed cell death. In cell death defective mutations, no programmed cell death should occur in the ventral epidermis, a region of the worm body that can easily be scored using differential interference contrast optics (Nomarski). We mutagenized wild-type animals with EMS, and analyzed the progeny of F_2 clonal lines by Nomarski optics. The progeny of approximately 3500 F_2 hermaphrodites were examined and three recessive mutations were isolated in which the ventral epidermal cells P(1-4).p and P(9-11).p survived (Fig. 3). P(1-4,8,9).p remain epidermal in such animals and P(5-7).p form a functional vulva (Fig. 6C). P11.p, and occasionally also P10.p differentiate in the pre-anal region. They can generate 6-8 progeny each by longitudinal cell divisions which in many animals do not detach from the cuticle. These features are cell lineage characteristics of P(10,11).p in males. In *C. elegans* males, P(10,11).p give rise to the hook and the pre-anal ganglion, structures used for mating with hermaphrodites (Sulston et al., 1980). P(10,11).p are part of an equivalence group in *C. elegans* and cell patterning is thought to depend on external signaling (Emmons and Sternberg, 1997). One simple interpretation for the differentiation of P(10,11).p in *P. pacificus* cell death mutants is that the signal that specifies the male equivalence group shows no sex-specific regulation in *P. pacificus*. Sex-specific regulation of this signal would not be necessary because P(9-11).p die in *P. pacificus* wild-type hermaphrodites and could therefore not respond to such a signal.

The three mutations fall into two complementation groups, which were originally named *Ppa-ipa-1* and *Ppa-ipa-2* for **i**nhibitor of **P**-ectoblast **a**poptosis. However, further phenotypic characterization indicated that the absence of cell death is not restricted to the ventral epidermal region. No cell death events were observed during embryonic and other aspects of postembryonic development. Thus, *Ppa-ipa-1* and *Ppa-ipa-2* are 'general' cell death defective mutations and are good candidates for being the homologs of *ced-3* and *ced-4*, which produce similar phenotypes in *C. elegans*.

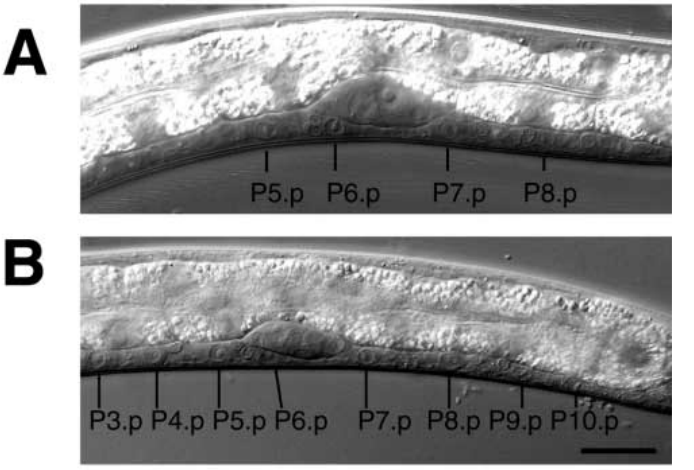

Fig. 3. Phenotype of cell death mutations in the ventral epidermal region. Pictures are Nomarski photomicrographs of L2 stages of the region covering P(3-10).p. (A) *P. pacificus* wild-type animal showing the four cells P(5-8).p. P(3,4,9,10).p are not present, because they underwent programmed cell death. (B) *P. pacificus* cell death defective animal of the strain *Ppa-ipa-1(tu79)*. P(3-10).p are visible in the central region. Scale bar, 20 μm.

Molecular cloning of the *P. pacificus* homolog of *ced-3*

The *C. elegans ced-3* gene is one of two genes that play an essential role in the regulation of the cell death program, since recessive mutations prevent almost all of the cell deaths that normally occur during *C. elegans* development (reviewed by Hengartner, 1997). *ced-3* encodes a cysteine protease with high sequence similarity to the human and murine interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993).

To clone the *P. pacificus* homolog of *ced-3*, we performed PCR experiments using degenerate primers to the highly conserved carboxy-terminal portion of the *C. elegans ced-3* gene (Fig. 4). This PCR fragment covers amino acids 250-283 (Figs 4, 5) and was used to screen a genomic and a cDNA library. None of the isolated cDNA clones was full length. To isolate a full length cDNA we made use of the fact that *C. elegans ced-3* is trans-spliced to an SL-1 leader sequence. We performed RT-PCR using an SL-1 specific primer in the 5′-region of the gene and a second primer from the 3′ region that had already been sequenced and thereby isolated a full length clone (see Materials and Methods for details). This PCR product contained an additional 350 base pairs covering the amino-terminal portion of *Ppa-ced-3*. Conceptual translation starts with the first in-frame ATG just 3 nucleotides behind the SL-1 leader. Genomic sequence analysis of this region revealed that the SL-1 leader is trans-spliced to a 3′splice acceptor site with a splice consensus sequence 5′-AUUGCAG-3′. *Ppa-ced-3* thereby confirms the presence of SL1 splicing in *P. pacificus*

which was first seen in *Ppa-mab-5* (Jungblut and Sommer, 1998).

The genomic organization of *Ppa-ced-3* differs from *Cel-ced-3* with respect to the exon-intron structure (Fig. 4). *Ppa-ced-3* contains 10 introns whereas *Cel-ced-3* contains only seven introns (Fig. 4A). The comparison of *Ppa*-CED-3 and *Cel*-CED-3 indicates that amino acid sequence similarity is lower in the amino-terminal portion. The carboxy-terminal portion of *Ppa*-CED-3, corresponding to amino acids position 170-418 is 47% identical to *Cel*-CED-3. Fig. 5 shows a sequence comparison of *Ppa*-CED-3 with *Cel*-CED-3 (Yuan et al., 1993) and the human ICE protein (Cerretti et al., 1992; Thornberry et al., 1992). The key residues within the active site of the caspases are highly conserved among the three proteins. From the human ICE and the *Cel*-CED-3 proteins it is known that they are cleaved at certain aspartic acid residues (see Fig. 5) all of which are in regions of low sequence similarity (Xue et al., 1996). Nonetheless, *Ppa*-CED-3 contains several aspartic acid residues in the corresponding region of the protein which might be used as cleavage sites in *P. pacificus*. Further evidence for these potential cleavage sites comes from computer analysis. Crystal structure analysis of the human ICE protein has indicated that the core of the protein starts with an α-helix, which is supposed to protect the protein (Wilson et al., 1994; Walker et al., 1994). Computer analysis of *Ppa*-CED-3 indicates that an α-helix can be formed in the corresponding region of the protein (Fig. 5). Taken together, the sequence comparison of *Ppa*-CED-3 with *Cel*-CED-3 and human ICE indicates that these proteins might have similar biochemical properties.

Ppa-ipa-1 is identical to Ppa-ced-3

To determine whether *Ppa-ipa-1(tu79)* or *Ppa-ipa-2(tu80, tu81)* correspond to mutations in *Ppa-ced-3*, we sequenced the coding region of *Ppa-ced-3* in the *Ppa-ipa-1* and *Ppa-ipa-2* mutants. This sequence analysis showed that the strain *tu79* contains a point mutation at Pro415 resulting in a missense mutation (Fig. 4B). In a suppressor screen (described below in more details) we isolated 16 additional alleles of this gene and sequence analysis of 5 of these alleles all identified point mutations. The point mutations in *tu84* and *tu104* result in nonsense mutations at Trp355 and Gln107 respectively (Fig. 4B; Table 1). Mutations in the alleles *tu90*, *tu103* and *tu105* result in missense mutations (Fig. 4B, Table 1) and the amino acids changed in *tu90* and *tu103* are conserved among *ced-3*-like molecules (Fig. 5). These results indicate that the *Ppa-ipa-1* mutant phenotye results from mutations in *Ppa-ced-3*. Therefore, we rename *Ppa-ipa-1* as

B

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                                                                  TAATTACCCAAGTTTGAGAT    20

ATGGCTGGTTCGATGCAGAATGAGCATCGAAAAGCCTTAATTGGATTCGGTGATTCGTCCCTTCCTCCACTCTAT    95
M  A  G  S  M  Q  N  E  H  R  K  A  L  I  G  F  G  D  S  S  L  P  P  L  Y    25

CTCGATCCTATTCTAGATTATCTTGAAGTGAAACAAGTACTACCTGAATCGAAGATTAACGAAATACGACGTATG   170
L  D  P  I  L  D  Y  L  E  V  K  Q  V  L  P  E  S  K  I  N  E  I  R  R  M    50

TCGAATGGCCGATTGGATCTAATGAGGAGTCTGACCAAACAGGGATCTATTGCATTCGATGAATTCTTCCTATCA   245
S  N  G  R  L  D  L  M  R  S  L  T  K  Q  G  S  I  A  F  D  E  F  F  L  S    75

CTCGTTCACTCACAACAAGTGCATCTGGCTGATTCCCTCCGACCACTCGTATCTCAGAGTGTCATCGCTAGTCTA   320
L  V  H  S  Q  Q  V  H  L  A  D  S  L  R  P  L  V  S  Q  S  V  I  A  S  L    100
                   tu104
GAGAACAAAGGAACGGAACAGAAAGCATCCAGTTCAATTGAGGGATTCGAGAGAATGGAAATTGATGAGGAAAGA   395
E  N  K  G  T  E  Q  K  A  S  S  S  I  E  G  F  E  R  M  E  I  D  E  E  R    125

GCAGGAGGAGGAGGAAGATATGATAGTGATGGTAATGGTAAATCTTCATTTTCTTCCTTTGATCTTCGTGATATT   470
A  G  G  G  G  R  Y  D  S  D  G  N  G  K  S  S  F  S  S  F  D  L  R  D  I    150

GAGAAGAAACTGGCATCGTATGAAGCACAGAAAGGAGAAAGAGTGTATCAGTTCACTTCTCCTCGAGGTTACGCT   545
E  K  K  L  A  S  Y  E  A  Q  K  G  E  R  V  Y  Q  F  T  S  P  R  G  Y  A    175
                                  tu103
CTTATCATATCCAATGAACATTTCCGGACGATGCCCCAGAGAAGAGGAACAAAAGCTGATGTTATTGCTCTTAAA   620
L  I  I  S  N  E  H  F  R  T  M  P  Q  R  R  G  T  K  A  D  V  I  A  L  K    200

AATCTGTTCATCTCTAAACTTGGATACGAAGTGGAAGTACATGAGAATCTAACTGGAAAAGAACTGCATAACAAT   695
N  L  F  I  S  K  L  G  Y  E  V  E  V  H  E  N  L  T  G  K  E  L  H  N  N    225
                                              tu105
ATCAAGTCATTCGCTTCTTCGACTAAACATTCTTTCACTTCATCTGCCATTGTCTGTATTCTCACTCATGGGGAA   770
I  K  S  F  A  S  S  T  K  H  S  F  T  S  S  A  I  V  C  I  L  T  H  G  E    250

CACGATGTTCTCTATGGAGTAGATGATCAAGTGGTTGATGTCCATTATCTTCTCTCTTCATTCAATACTCATAAT   845
H  D  V  L  Y  G  V  D  D  Q  V  V  D  V  H  Y  L  L  S  S  F  N  T  H  N    275

GCACCTCATTTGGCAGGGAAACCAAAGATTATTCTCCTTCAAGCCTGTCGAGGAGAAAGGAAAGATATTGGATAT   920
A  P  H  L  A  G  K  P  K  I  I  L  L  Q  A  C  R  G  E  R  K  D  I  G  Y    300

GAAGAGACTGATTCTGGTCGACCATTTGCCTTTCTTGAATGTCTTGGAATGGGAGGAGGAGGAATGATGACTAGG   995
E  E  T  D  S  G  R  P  F  A  F  L  E  C  L  G  M  G  G  G  G  M  M  T  R    325
                        tu90
AATAAACGACCAGCCGAATCTGATATACTCGTTGCTTATGCAACTCCTGCCCATTACGTTAGTTGGAGGAACAGT  1070
N  K  R  P  A  E  S  D  I  L  V  A  Y  A  T  P  A  H  Y  V  S  W  R  N  S    350
          tu84
GAGAAGGGAAGCTGGTTTATTCAATCTTTTGTTGAGGTAGCGGAGAAGAGAAGTCACGATTCGGATCTCCTTTCT  1145
E  K  G  S  W  F  I  Q  S  F  V  E  V  A  E  K  R  S  H  D  S  D  L  L  S    375

ATTCTTACTCAAGTTAATGCTAAAGTAGCCGAGACTTACAAGGCTAATTCATCGACAGGAGAATACAAACAAATA  1220
I  L  T  Q  V  N  A  K  V  A  E  T  Y  K  A  N  S  S  T  G  E  Y  K  Q  I    400
                                        tu79
TCAGAATTTAGCTCCAAATTAACCAAGAAGTTCTTCTTCTTCCCTCCAGAATAGTCATTGATCCATGACCATGTA  1295
S  E  F  S  S  K  L  T  K  K  F  F  F  F  P  P  E  *                          417

CATATGTTCTTCTAGTCTCTTTTCCCCTTTTTATCGCTCTTGATTGTACAAATCTTTGATTCATTCTGCAAAATC  1370

TTTGGATGCTTTCATTGTACAGGGAATCAATCTCCCTTTCATTCACTGCCACTGTGATATCAATCGATTAATTTT  1445

GGATAAATCTTAGATGTCCAAA                                                        1467
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Fig. 4. Cloning and sequencing analysis of *Ppa-ced-3*. (A) *Ppa-ced-3* genomic structure. The gene contains 10 introns, 8 of which are less than 100 base pairs in length. The highly conserved pentapeptide containing the active cysteine in ICE proteins is shown as a black box. (B) *Ppa-ced-3* cDNA sequence as obtained from the sequence of cDNA library clones and from RT-PCR experiment described in the text and in the Materials and Methods. SL-1 leader sequence is in italics. Conceptual translation starts with the first in-frame ATG codon after the SL-1 splice acceptor site. The position of the *Ppa-ced-3* mutations is indicated by arrows (compare Table 1). Introns are indicated by triangles. The accession number of this sequence is AF071505.

Fig. 5. Amino acid sequence comparison between *Ppa*-CED-3, *Cel*-CED-3 (Yuan et al., 1993) and the human ICE protein (Cerretti et al., 1992; Thornberry et al., 1992). Identical amino acids are shaded in black, similar amino acids are shaded in grey. Dashes indicate gaps in the sequence to allow optimal alignment. *Ppa-ced-3* mutations are indicated below the comparison showing the residue in the mutant CED-3 and the allele name. The active site of the protein is underlined (QACRG, amino acid position 289-293 in *Ppa*-CED-3). The position of the human ICE and the *Cel*-CED-3 cleavage sites are indicated in bold (Xue et al., 1996). The α-helix at the start of the core of the human ICE protein is indicated by a bar above the sequence (labeled with α) (Wilson et al., 1994; Walker et al., 1994). α-helix secondary structure prediction was made using Garnier-Robson and Chou-Fasman algorithms which both predict that an α-helix can be formed in *Ppa*-CED-3 in the region of amino acid 146-163, which is carboxy-terminal to the potential cleavage sites. In general, *Ppa*-CED-3 and the human ICE protein are similar in size and the (potential) cleavage sites are in similar positions in both proteins. In contrast, the *Cel*-CED-3 protein is substantially longer. Sequence alignment was made using the Clustal analysis and visual inspection of the proteins.

Ppa-ced-3. The six *Ppa-ced-3* alleles that have been sequenced produce similar phenotypes. In nearly all mutant animals, the seven cells P(1-4,9-11).p survived.

Ppa-lin-39; Ppa-ced-3 double mutants have a functional vulva

To determine whether LIN-39 has a permissive or an instructive role and whether LIN-39 activity prevents the nonvulval cell fate (cell death) or promotes the vulval cell fate, double mutants have been generated between *Ppa-lin-39* and *Ppa-ced-3*. If *Ppa*-LIN-39 has a permissive role and prevents cell death without contributing to vulval cell fate specificity, such double mutants should have a functional vulva (Fig. 1D). However, if *Ppa*-LIN-39 has an instructive role and promotes vulval fate, such double mutants should be vulvaless, with P(5-7).p adopting an epidermal fate (Fig. 1D). We generated double mutants of the two *Ppa-ced-3* alleles *Ppa-ced-3(tu79)* and *Ppa-ced-3(tu84)* with the weak allele *Ppa-lin-39(tu29)* and the strong allele *Ppa-lin-39(tu2)*. In all double mutant combinations, animals have a normal functional vulva (Fig. 6D): cell lineage observation of double mutant animals revealed wild-type vulval lineages in 33 of 35 animals analyzed. In the two other animals, P(5,6).p had vulval cell lineages as in wild-type animals, whereas P7.p had an epidermal fate and did not divide. Taken together, these results suggest that the primary function of *Ppa*-LIN-39 is to permit vulval fate by blocking cell death.

Cel-lin-39 has a second role during vulva formation. *Cel*-LIN-39 directs vulval cell fates in the L3 stage in response to RAS signaling (Clandinin et al., 1997; Maloof and Kenyon, 1998). This situation differs from the one in *P. pacificus*. *Ppa-lin-39*; *Ppa-ced-3* double mutants have a normal vulva indicating that *Ppa*-LIN-39 inhibits programmed cell death early in development, but is not reused later on in response to gonad induction. Thus, *Ppa-lin-39* is dispensable during vulval induction.

Table 1. Sequences of *ced-3* mutations

Allele	Wild-type sequence	Mutant sequence	Substitution
tu79	CCA	TCA	P416S
tu84	TGG	TAG	W355STOP
tu90	CTC	TTC	L335F
tu103	GGA	GAA	G191E
tu104	CAG	TAG	Q107STOP
tu105	TGT	TAT	C244Y

Amino acid positions correspond to the numbering in Fig. 4.

Suppression of the *Ppa-lin-39* vulvaless phenotype by cell death mutants as a search for cell death signals

Ppa-lin-39(tu29) mutant animals are vulvaless and egg-laying defective, whereas *Ppa-lin-39; Ppa-ced-3* double mutants have a functional vulva and they are egg-laying positive. This observation can be used to isolate cell death mutants in a *Ppa-lin-39* suppressor screen. In a mutagenesis screen in a *Ppa-lin-39* mutant background (egg-laying defective), suppressors can be identified by the reappearance of eggs (egg-laying positive). Such a screen can be performed to isolate general cell death defective mutations, but also to isolate genes that affect the programmed death of only certain cells. Such 'specific' cell death genes might be involved in the regulation of the cell death in the ventral epidermal region but not in the regulation of the death of other cells that die in wild-type animals. Such genes might act like a local 'killer' signal which activates the

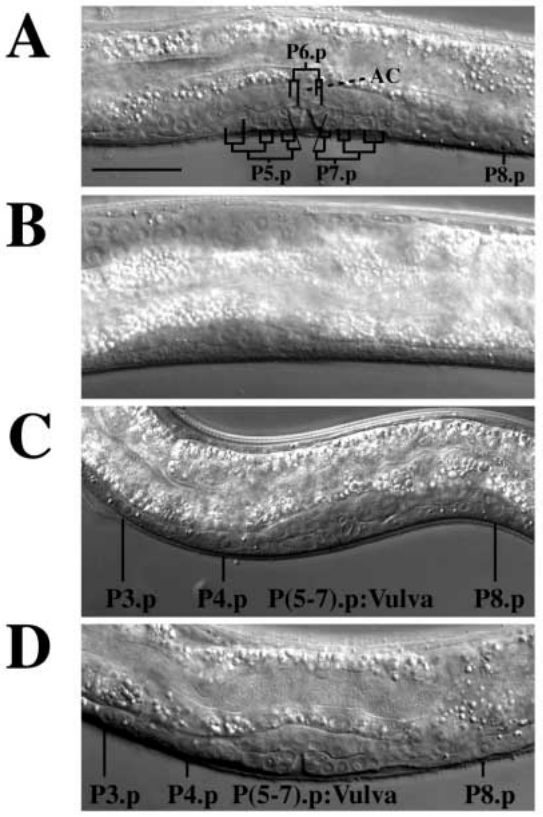

Fig. 6. Phenotype of *Ppa-lin-39*, *Ppa-ced-3* and *Ppa-lin-39; Ppa-ced-3* double mutant animals. All pictures are Nomarski photomicrographs of the central body region corresponding to P(3-8).p in the early L4 stage. (A) *P. pacificus* wild-type animal. P(3,4).p are absent. The vulva cell lineage of P(5-7).p is indicated. P(5,7).p have completed their lineage, P6.p underwent only two rounds of cell division in this stage. P8.p is epidermal. AC, anchor cell. (B) *Ppa-lin-39(tu2)* mutant, P(5-8).p are absent. This animal is in the mid L4 stage and no vulva is present. The AC is not visible in this plane of focus. (C) *Ppa-ced-3(tu79)* mutant. P(5-7).p underwent two rounds of cell division, P(3,4).p are epidermal. (D) *Ppa-lin-39(tu2); Ppa-ced-3(tu79)* double mutant with a normal vulva formed by P(5-7).p. P(3,4).p are epidermal. Scale bar, 20 μm.

cell death machinery in P(1-4,9-11).p. As egg-laying screens are not very labour intensive, one can perform large-scale screens and thus, one might be able to saturate for potential 'specific cell death' genes.

To look for the existence of such a killer signal in the *P. pacificus* ventral epidermis, we have carried out a suppressor screen in the *Ppa-lin-39(tu29)* mutant background. We have EMS-mutagenized approximately 60 000 gametes and have isolated 16 suppressors that are egg-laying positive. Nomarski observation indicated that in all 16 suppressors all 12 Pn.p cells are present as in the originally described *Ppa-ced-3(tu79)* and *Ppa-ipa-2* mutants. P(1-4).p and P(8,9).p have an epidermal fate, P(5-7).p form the vulva and P11.p and sometimes P10.p differentiate in the pre-anal region. After outcrossing, these suppressors keep the cell death defective phenotype. Genetic complementation tests revealed that all 16 suppressors are allelic to *Ppa-ced-3*. This has been confirmed by identifying molecular lesions in the *Ppa-ced-3* gene in the five mutations *tu84*, *tu90*, *tu103*, *tu104* and *tu105* (Fig. 4B; Table 1). Complementation tests between the suppressor *Ppa-ced-3(tu104)* that results in a nonsense mutation at amino acid Q107 (Fig. 4) and *Ppa-ipa-2(tu80)* generated wild-type progeny indicating that these mutant strains belong to different genes. In summary, these results indicate that the *Ppa-lin-39* suppressor screen has a strong bias towards the isolation of *Ppa-ced-3* alleles. In addition, there is no indication that a killer signal acts to specifically induce the cell death of P(1-4,9-11).p and thus, no 'specific cell death' genes have been identified.

DISCUSSION

We have described the genetic and molecular characterization of *Ppa-ced-3*, a regulator of programmed cell death in *P. pacificus*. In *Ppa-ced-3* mutant animals, no programmed cell death is observed during embryogenesis and postembryogenesis. Like *Cel-ced-3*, *Ppa-ced-3* is not essential for viability as *Ppa-ced-3* mutant animals are homozygous viable. We have identified molecular lesions in six of the *Ppa-ced-3* mutations, at least some of which are likely to eliminate *Ppa-ced-3* activity.

The primary function of *Ppa-lin-39* is the inhibition of programmed cell death

We have addressed the question of the mechanism of *lin-39* function in *P. pacificus* by studying double mutants between *Ppa-lin-39* and *Ppa-ced-3*. In *P. pacificus* wild-type animals, the non-vulval cells P(1-4,9-11).p undergo programmed cell death, whereas in *Ppa-lin-39* mutants also the central cells P(5-8).p die of apoptosis. When we generated double mutants between *Ppa-lin-39* and *Ppa-ced-3*, the phenotype of such double mutants provided direct evidence that the primary function of *Ppa-lin-39* during the formation of the vulva equivalence group is the inhibition of programmed cell death of P(5-8).p. In the absence of programmed cell death, *Ppa-lin-39* is not required to establish the vulva equivalence group and such double mutants have a functional vulva. Thus, *Ppa-lin-39* has a permissive function by inhibiting programmed cell death of the central Pn.p cells.

By analogy, this result suggests that *Cel-lin-39* might also have a permissive function by repressing cell fusion of the *C. elegans* VPCs. It remains unknown however, if *lin-39* directly or indirectly regulates cell fate in the two nematode species. One model would be that LIN-39 regulates different target genes in both species which subsequently interact with the cell fusion and the cell death machinery respectively. Alternatively, LIN-39 might regulate identical target genes, which only later interact with different regulatory machineries.

Recently, Félix and Sternberg (1998) have shown that a gonad-derived survival signal for the VPCs exists in *Turbatrix aceti* and *Halicephalobos* sp. JB128. In contrast to *C. elegans*, *P. pacificus* and many other nematode species, ablation of the gonad in the L1 stage of both these species causes all VPCs to undergo programmed cell death (Félix and Sternberg, 1998). Although the involvement of a *lin-39* homolog in fate specification of the VPCs has not been demonstrated in *T. aceti* and *Halicephalobus* sp. JB128, one could speculate that the gonad-derived survival signal acts to regulate LIN-39 activity. In *P. pacificus*, such a survival signal is, if present at all, not

provided by the gonad. However, we cannot rule out that other cells provide a survival factor for the VPCs that in turn regulates LIN-39 activity.

Ppa-lin-39 is not used in response to gonad induction

Cel-lin-39 is required during vulval induction to specify the outcome of Ras signaling. The Ras pathway, together with basal *Cel-lin-39* activity, up-regulates *Cel-lin-39* expression in VPCs (Maloof and Kenyon, 1998). If *Cel-lin-39* function is absent at that time vulva formation does not take place. In experiments in which *Cel-lin-39* has been replaced by the posterior Hox gene *Cel-mab-5*, the VPCs express posterior cell fate identity indicating further that *Cel-lin-39* selectively activates vulva-specific genes and thereby provides specificity to Ras signaling (Maloof and Kenyon, 1998).

In *P. pacificus*, vulva formation is also induced by gonadal signaling. If Z(1,4), the precursors of the somatic gonad, are ablated P(5-7).p do not divide and no vulva is formed (Sommer and Sternberg, 1996). However, evidence from the *Ppa-lin-39; Ppa-ced-3* double mutant described here, argues clearly against a role of *Ppa-lin-39* in response to gonadal signaling (Fig. 6D). Such double mutants form a normal vulva indicating that *Ppa*-LIN-39 is dispensable for vulva formation if programmed cell death is blocked early in development. Thus, the second function of *Cel-lin-39* during vulva development has changed dramatically during evolution. This result raises the intriguing possibility that the entire EGF/Ras/MAPK pathway is not involved in vulval induction in *P. pacificus*. However, two alternative hypotheses could also account for the observed results. First, *lin-39* might be the only transcription factor acting downstream of EGF/Ras/MAPK signaling that changed its function during evolution. For example, *lin-39* might have been replaced by another unknown transcription factor in *P. pacificus* which is still under the control of EGF/Ras/MAPK signaling. Alternatively, the gene *lin-39* might have been duplicated in *P. pacificus* with one gene specifying the vulval equivalence group and the other one responding to EGF/Ras/MAPK signaling. However, so far we have no evidence for a duplication of *lin-39* in *P. pacificus*.

Evolution of gene function

Cell lineage analysis indicated that the vulvae of *C. elegans* and *P. pacificus* are homologous structures. In both species the vulva is formed from a set of three homologous precursor cells P(5-7).p. Therefore, the differential use of LIN-39 during vulval induction suggests that during evolution, individual components of genetic systems have changed their function in a homologous developmental/morphological structure. Thus, the mechanisms of evolutionary change of developmental systems include differential combinatorial coding of genetic systems. Theoretically, differential combinatorial coding can occur at the level of genes (within genetic pathways) or at the level of complete genetic pathways (within genetic networks). We speculate that redundancy in genetic pathways and networks will facilitate this type of evolutionary alterations. In summary, the case of the nematode Hox gene *lin-39* is a prominent example for co-option (Raff, 1996), a phenomenon describing the fact that individual genes can jump out and/or jump into specific genetic circuits. Further work on the nematode *lin-39* gene will show additional molecular requirements for the occurrence of co-option during the evolution of genetic circuits.

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REFERENCES

Aroian, R. V., Koga, M., Mendel, J. E., Ohshima, Y. and Sternberg, P. W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**, 693-699.

Beitel, G. J., Clark, S. G. and Horvitz, H. R. (1990). *Caenorhabditis elegans* ras gene *let-60* acts as a switch in the pathway of vulva induction. *Nature* **348**, 503-509.

Beitel, G. J., Tuck, S., Greenwald, I. and Horvitz, H. R. (1995). The *Caenorhabditis elegans* gene *lin-1* encodes an ETS domain protein and defines a branch of the vulva induction pathway. *Genes Dev.* **9**, 3149-3162.

Cerretti, D. P., Kozlosky, C. J., Mosley, B. et al. (1992). Molecular cloning of the interleukin-1β converting enzyme. *Science* **256**, 97-100.

Clandinin, T. R., Katz, W. S., and Sternberg, P. W. (1997). *Caenorhabditis elegans* HOM-C genes regulate the response of vulval precursor cells to inductive signal. *Dev. Biol.* **182**, 150-162.

Clark, S. G., Chisholm, A. D. and Horvitz, H. R. (1993). Control of cell fates in the central body region of C. elegans by the homeobox gene *lin-39*. *Cell* **74**, 43-55.

Emmons, S. W. and Sternberg, P. W. (1997). Male development and mating behavior. In *C. elegans II* (eds. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 295-334. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Eizinger, A. and Sommer, R. J. (1997). The homeotic gene lin-39 and the evolution and nematode epidermal cell fates. *Science* **278**, 452-455.

Epstein, H. F. and Shakes, D. C. (1995). *Caenorhabditis elegans: Modern biological analysis of an organism*. Methods in Cell Biology. Volume 48. Academic Press. San Diego.

Félix, M.-A. and Sternberg, P. W. (1998). A gonad-derived survival signal for vulval precursor cells in two nematode species. *Curr. Biol.* **8**, 287-290.

Han, M. and Sternberg, P. W. (1990). *let-60*, a gene that specifies cell fates during C. elegans vulval induction encodes a ras protein. *Cell* **63**, 921-931.

Hengartner, M. O. (1997). Cell Death. In *C. elegans II* (eds. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 295-334. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Hill, R. J. and Sternberg, P. W. (1992). The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans. Nature* **358**, 470-476.

Jungblut, B. and Sommer, R. J. (1998). The Pristionchus pacificus *mab-5* gene is involved in the regulation of ventral epidermal cell fates. *Curr. Biol.* **8**, 775-778.

Kimble, J. (1981). Lineage alterations after ablation of cells in the somatic gonad of *C. elegans. Dev. Biol.* **87**, 286-300.

Kornfeld, K. (1997). Vulval development in *Caenorhabditis elegans*. *Trends Genet.* **13**, 55-61.

Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.

Lackner, M. R., Kornfeld, K., Miller, L. M., Horvitz, H. R. and Kim, S. (1994). A MAP kinase homolog, *mpk-1*, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans. Genes Dev.* **8**, 160-173.

Lawrence, P. A. and Morata, G. (1994). Homeobox genes: their function in Drosophila segmentation and pattern formation *Cell* **78**, 181-189.

Maloof, J. N. and Kenyon, C. (1998). The HOX gene *lin-39* is required during *C. elegans* vulval induction to select the outcome of Ras signaling. *Development* **125**, 181-190.

Raff, R. A. (1996). *The shape of life*. The University of Chicago Press. Chicago.

Salser, S. J. and Kenyon, C. (1994). Patterning *C. elegans*: homeotic cluster genes, cell fates and cell migrations. *Trends Genet.* **10**, 159-164.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning. Cold Spring Harbor Laboratory Press. Cold Spring Harbor.

Schlötterer, C. and Wolff, C. (1996). M13mtvh: an improved M13 vector for rapid and simple cloning of PCR products. *Trends Genet.* **12**, 286-287.

Sommer, R. J., Carta, L. K., Kim, S.-Y. and Sternberg, P. W. (1996). Morphological, genetic and molecular description of *Pristionchus pacificus* sp. n. (Nematoda: Neodiplogastridae). *Fund. Appl. Nemat.* **19**, 511-521.

Sommer, R. J. and Sternberg, P. W. (1996). Apoptosis and change of competence limit the size of the vulva equivalence group in *Pristionchus pacificus*: a genetic analysis. *Curr. Biol.* **6**, 52-59.

Sulston, J. E. and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **56**, 110-156.

Sulston, J. E., Albertson, D. G. and Thomson, J. N. (1980). The *Caenorhabditis elegans* male: Postembryonic development of nongonadal structures. *Dev. Biol.* **78**, 542-576.

Thorberry, N. A., Bull, H. G., Calaycay, J. R. et al. (1992). A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature* **356**, 768-774.

Walker, N. P. C., Talanian, R. V., Brady, K. D., et al. (1994). Crystal structure of the cysteine protease Interleukin-1β-converting enzyme: A (p20/p10)2 homodimer. *Cell* **78**, 343-352.

Wang, B. B., Müller-Immerglück, M. M., Austin, J., Robinson, N. T., Chisholm, A. and Kenyon, C. (1993). A homeotic gene cluster patterns the anteroposterior body axis of C. elegans. *Cell* **74**, 29-42.

Wilson, K. P., Black, J.-A. F., Thomson, J. A. et al. (1994). Structure and mechanism of interleukin-1β converting enzyme. *Nature* **370**, 270-2275.

Wu, Y. and Han, M. (1994). Suppression of activated let-60 ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. *Genes Dev.* **8**, 147-159.

Xue, D., Shaham, S. and Horvitz, H. R. (1996). The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of human CPP32 protease. *Genes Dev.* **10**, 1073-1083.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. and Horvitz, H. R. (1993). The C. elegans cell death *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell* **75**, 641-652.