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; Cladnin 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 (Cladnin 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 vulvalless, 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.

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, F2 progeny of the mutagenized animals were picked individually to plates and their progeny were analyzed under Nomarski optics. Usually, 10 F3 animals were analyzed per F2 parent. Altogether, 3500 clonal F2 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 vulvalless, 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 Psp 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'-CTGGTCTATYTRWCNGYGG-3' and RS127, 5'-CTGGAC-RAAGACRTYTTNGGTTG-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-Reverse Transcriptase (Stratagene Cloning Systems, La Jolla, CA) and the primer RS202, 5'-GAGCTCCTTCATCCATA-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'-GGTTTACCATACCCAGTTTGGG-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'-AGAATACACAGATGGGAAGGA-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). The allele Ppa-lin-39(tu29) was found to represent a strong allele, where on average only 0.01 VPCs survive per animal. The allele Ppa-lin-39(tu29) was found to represent a stronger allele, where on average 0.09 VPCs survive per animal.

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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, AC signaling inactivates lin-1 which functions to inhibit vulva formation (Beitel et al., 1995). In lin-1 mutants, P(5-8).p undergo cell fusion like their anterior and posterior neighbors. (C) 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. 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.

**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 a Ras molecule acting as a molecular switch (Han and Sternberg, 1990; Beitel et al., 1990), mpk-1/sur-1 encodes a MAP 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) (Somer 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 mutants, 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 F2 clonal lines by Nomarski optics. The progeny of approximately 3500 F2 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). P(11),p and occasionally also P(10),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 inhibitor of P-ectoblast apoptosis. 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*.

**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., 1994; Walker et al., 1994). 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 phenotype results from mutations in *Ppa-ced-3*. Therefore, we rename *Ppa-ipa-1* as
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 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 vulvalless, 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 P(7).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.

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

Ppa-lin-39(tu29) mutant animals are vulvalless 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 mutants, 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 death 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
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 *Halicephalobus* 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 provided by the gonad. 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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