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Article

HAIRY-like Transcription Factors and the Evolution of the Nematode Vulva Equivalence Group

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Summary

Background: Nematode vulva formation provides a paradigm to study the evolution of pattern formation and cell-fate specification. The *Caenorhabditis elegans* vulva is generated from three of six equipotent cells that form the so-called vulva equivalence group. During evolution, the size of the vulva equivalence group has changed: *Panagrellus redivivus* has eight, *C. elegans* six, and *Pristionchus pacificus* only three cells that are competent to form vulval tissue. In *P. pacificus,* programmed cell death of individual vulval precursor cells alters the size of the vulva equivalence group.
Results: We have identified the genes controlling this cell-death event and the molecular mechanism of the reduction of the vulva equivalence group. Mutations in *Ppa-hairy*, a gene that is unknown from *C. elegans*, result in the survival of two precursor cells, which expands the vulva equivalence group. Mutations in *Ppa-groucho* cause a similar phenotype. *Ppa*-HAIRY and *Ppa*-GROUCHO form a molecular module that represses the Hox gene *Ppa-lin-39* and thereby reduces the size of the vulva equivalence group. The *C. elegans* genome does not encode a similar *hairy*-like gene, and no typical HAIRY/GROUCHO module exists.
Conclusions: We conclude that the vulva equivalence group in *Pristionchus* is patterned by a HAIRY/GROUCHO module, which is absent in *Caenorhabditis*. Thus, changes in the number, structure, and function of nematode *hairy*-like transcription factors are involved in the evolutionary alteration of this equivalence group.

Introduction

Vulva formation in *Caenorhabditis elegans* provides a molecular framework for studying pattern formation, cell-fate specification, developmental competence, and induction. Interestingly, all of these aspects of vulva formation are subject to evolutionary change. To identify the genetic and molecular alterations that control evolutionary changes of developmental processes, different species have to be compared with one another. The model organism *C. elegans* and the satellite species *P. pacificus* are both amenable to forward and reverse genetic analysis and provide a platform for functional comparative studies [1–4]. *P. pacificus* propagates as a self-fertilizing hermaphrodite and has a 4 day life cycle at 20°C. An integrated genome map of *P. pacificus* contains a genetic linkage map of more than 500 molecular markers and a physical map of nearly 10,000 fingerprinted BAC clones [3–5]. A whole-genome sequencing project is currently ongoing (http://www.nhgri.nih.gov/12511858).

The nematode vulva is a derivative of the ventral epidermis, which consists of 12 precursor cells, called P(1–12).p, in all nematodes studied to date (Figure 1A). In *C. elegans,* developmental competence and induction are the two key features of vulva formation. First, a group of six equipotent cells is specified as a vulva equivalence group (VEG). Second, vulva induction from the gonadal anchor cell (AC) selects three of these six cells to form vulval tissue [6]. Molecularly, the HOX gene *lin-39* defines developmental competence and establishes P(3–8).p as vulval precursor cells (VPC) (Figure 1B) [7, 8]. The epidermal growth factor (EGF)-like molecule LIN-3 is secreted from the AC and induces P(5–7).p to adopt one of two alternative vulval fates. P6.p has the so-called 1° fate, generates eight progeny, and forms the central part of the vulva. P(5,7).p have the 2° fate, generate seven progeny each, and form the outer part of vulva. The three other VPCs, P(3,4,8).p, have an epidermal (3°) fate (Figure 1B). After cell ablation of P(5–7).p, P(3,4,8).p can substitute for the other VPCs.

The size of the VEG varies greatly among nematodes (Figure 1B) [9]. The largest VEG described to date is that of *P. redivivus* of the Panagrolaimidae family, consisting of eight cells, four of which participate in vulva formation in wild-type animals (Figure 1B) [10, 11]. Of the four additional competent cells, two are found in the anterior and two in the posterior region. Compared to *P. redivivus*, the VEG of *C. elegans* and other species of the Rhabditidae family is altered in the posterior region and consists of five or six cells [12, 13]. In *P. pacificus* and its relatives, the VEG shows an additional alteration in the anterior body region. Only those cells that eventually will form vulval tissue are able to respond to the inductive signal from the somatic gonad [14]. Specifically, the programmed cell death of P3.p and P4.p in *P. pacificus* causes a reduction in the size of the VEG.

C. elegans itself is an unusual nematode with regard to the size of the VEG. The cell lineage of the anterior-most cell of the VEG, P3.p, is variable within genetically homogenous populations and is polymorphic between wild isolates [15]. In the laboratory strain N2, P3.p has a 3° fate in 50% of the animals, whereas it is not part of the VEG and fuses with the hypodermis in the remaining 50% of the animals. Interestingly, several genes that are involved in the regulation of vulva induction also influence the P3.p cell fate decision. For example, gain-of-function mutations in *let-60/ras* and loss-of-function mutations in the synMuvA and synMuvB pathways expand the VEG and cause ectopic differentiation of P3.p in nearly 100% of mutant animals [16]. Besides those genes that have a role in the regulation of vulva

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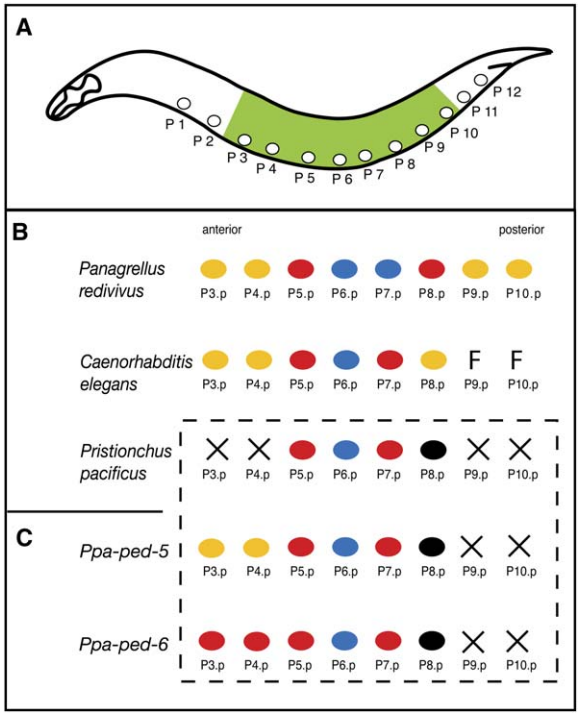

Figure 1. Evolution of the Size of the Vulva Equivalence Group

(A) Schematic summary of nematode vulva development. The ventral epidermis derives from 12 ectoblasts, named P(1–12) according to their anteroposterior position.

(B) Schematic summary of the cell fate of ventral epidermal cells in *P. redivivus*, *C. elegans*, and *P. pacificus*. The vulva is formed from the progeny of the 1° (blue ovals) and 2° (red ovals) vulva precursor cells. 3° cells (yellow ovals) are competent to form vulval tissue but remain epidermal under wild-type conditions. In *Panagrellus redivivus*, the vulva is formed from P(5–8).p with two additional competent cells in the anterior and posterior body region, respectively. In *C. elegans*, P(9,10).p fuse (F) with the hypodermis and are not competent to form part of the vulva. In *P. pacificus*, P(3,4).p die of programmed cell death (X) and further reduce the size of the vulva equivalence group.

(C) Phenotype of *Ppa-ped-5* and *Ppa-ped-6* mutants expanding the vulva equivalence group in the anterior body region. In *ped-5*, P(3,4).p survive and remain epidermal, whereas in *ped-6*, P(3,4).p survive and differentiate ectopically (red ovals).

induction, mutations in *Cel-lin-22* also bring P3.p into the VEG. *Cel-lin-22* encodes a bHLH molecule of the HAIRY family, and in *Cel-lin-22* mutants, P3.p is a member of the VEG in 100% of the animals [17].

We have used a genetic approach to study the evolution of the size of the VEG by screening for mutants that expand the VEG in *P. pacificus*. We show that changes in number, domain structure, and function of nematode *hairy*-like transcription factors are involved in the evolutionary restriction of the equivalence group. In *P. pacificus*, mutations in a *hairy* and a *groucho* gene cause the survival of P(3,4).p and expand the size of the VEG. We show that *Ppa*-HAIRY and *Ppa*-GROUCHO repress the Hox gene *Ppa-lin-39* and thereby reduce the size of the equivalence group. In *C. elegans*, no typical HAIRY/GROUCHO module exists. Thus, changes in the number, structure, and function of nematode *hairy*-like transcription factors are involved in the evolutionary alteration of the vulva equivalence group.

Table 1. Genetic Regulation of P(3,4).p Survival and Differentiation in *P. pacificus*

	Genotype	%P3.p S	%P4.p S	%P3.p D	%P4.p D	n
1	Wild-type PS312	0	0	n.a.	n.a.	many
2	*Ppa-hairy(sy344)*	100	100	0	0.5	400
3	*Ppa-hairy(tu96)*	100	100	0	0	70
4	*Ppa-hairy(tu97)*	100	100	0	0	73
5	*Ppa-hairy(sy344); Ppa-groucho(tu102)*	100	100	55	76	38
6	*Ppa-lin-39(tu29)*	0	0	n.a.	n.a.	60
7	*Ppa-hairy(sy344); Ppa-lin-39(tu29)*	8	29	0	0	24
8	*Ppa-hairy(sy344); Ppa-mab-5(tu357)*	100	100	0	0	53
9	*Ppa-groucho(tu102)*	100	100	61	71	28
10	*Ppa-groucho(tu102); Ppa-lin-39(tu29)*	16	40	n.a.	n.a.	25

The P(3,4).p survival (S) and ectopic differentiation (D) were measured by cell-lineage analysis. P(3,4).p survival was analyzed in the late J2 stage, differentiation in the late J3/early J4 stage. For the *Ppa-groucho(tu102); Ppa-lin-39(tu29)* double mutant, differentiation cannot be scored unambiguously because surviving P3.p and P4.p cells migrate towards the center and replace the missing core VPCs.

Results

Mutations in *ped-5* and *ped-6* Expand the VEG in *P. pacificus*

To study the molecular basis of evolutionary changes that result in novel structures, it is essential to trace the activity of individual genes. To analyze the genetic regulation of the reduction of the VEG in the anterior body region in *P. pacificus* relative to *C. elegans,* we screened for *P. pacificus* mutants that exhibit an extension of the VEG. In various mutagenesis screens, we identified multiple alleles of two genes that, when mutated, expand the VEG. Specifically, mutations in *ped-5* and *ped-6* inhibit the programmed cell death of P3.p and P4.p without affecting apoptosis of P(1,2,9–11).p (Figure 1C, Tables 1 and 2) [1]. Interestingly, the cell fate of the two surviving cells differs between *ped-5* and *ped-6*. In *ped-5* mutants, P(3,4).p survive, remain epidermal, and are competent to adopt vulval fates. This phenotype is fully penetrant in all three alleles (Table 1, lines 1–5). In addition, P(3,4).p can form ectopic vulval tissue in the multivulva background of *Ppa-lin-17/Frizzled* [18]. Thus, the *ped-5* mutant reconstitutes the ancestral VEG and, by definition, represents an atavistic condition.

Mutations in the gene *ped-6* result in the survival of P(3,4).p and cause the ectopic differentiation of both cells into vulval tissue (Table 2). Although the survival phenotype of P(3,4).p is fully penetrant, ectopic vulva differentiation varies between both cells and the individual alleles. Usually, P4.p differentiates to a higher degree than P3.p (Table 2). The ectopic differentiation of P(3,4).p suggests that *ped-6* is a multivulva mutation, in which negative signaling is impaired as in *Ppa-lin-17/Frizzled* [18]. To test whether *ped-6* fulfills all the criteria of a multivulva gene, we ablated the gonad at hatching in eight of the *ped-6* alleles. Indeed, vulva differentiation by P(3–7).p was observed in all tested *ped-6* alleles (Table 2). Thus, *ped-6* has a dual role; it

Table 2. Phenotype and Molecular Lesions of the *ped-6*/*Ppa-groucho* Alleles

Allele	%P3.p S (n)	%P4.p S (n)	%P3.p D (n)	%P4.p D (n)	% Gid (n)	Lesion
tu40	100 (50)	100 (50)	62 (21)	81 (21)	98 (40)	Gln608OPAL
tu41	100 (50)	100 (50)	26 (97)	59 (97)	62 (50)	G to A acceptor site intron 17
tu43	100 (50)	100 (50)	55 (33)	70 (33)	63 (40)	not determined
tu44	100 (50)	100 (50)	43 (23)	74 (23)	66 (35)	G to A donor site intron 10
tu45	100 (50)	100 (50)	48 (21)	86 (21)	40 (30)	not determined
tu102	100 (50)	100 (50)	61 (28)	71 (28)	70 (20)	deletion parts of intron 9 to 10
tu141	100 (50)	100 (50)	14 (28)	75 (28)	69 (35)	deletion parts of intron 1 and exon 2
tu142	100 (50)	100 (50)	29 (24)	75 (24)	53 (40)	Gly348Arg

The P(3,4).p survival (S) and ectopic differentiation (D) were measured by cell-lineage analysis. P(3,4).p survival was analyzed in the late J2 stage, differentiation in the late J3/early J4 stage. Gonad-independent vulva differentiation (Gid) is measured after ablation of the gonadal precursor cells Z1 and Z4 at hatching. Numbers in parentheses represent number of analyzed cells.

specifically regulates the cell death of P(3,4).p and acts as a negative signal to prevent *P. pacificus* vulva formation.

ped-5 Encodes a *hairy*-like Transcription Factor that Does Not Exist in *C. elegans*

To identify the molecular nature of *ped-5*, we mapped the locus by using the polymorphic reference strain *P. pacificus var.* Washington [3]. *ped-5* maps to the center of chromosome IV between the molecular markers S115 and S2. Another marker that maps to the same region of the genome is S332, which is associated with a *hairy*-like transcription factor (Figure 2A). When we sequenced the complete *hairy*-like gene as a candidate for *ped-5,* we found that it is mutated in all alleles of *ped-5* (Figures 2B and 2C). We identified mutations resulting in stop codons of W120 in *tu96* and *sy344* and an amino acid replacement of R51S in *tu97* (Figures 2B and 2C). We conclude that *ped-5* is *Ppa-hairy*. *Ppa-hairy* has a bona fide bHLH domain and a GROUCHO interaction domain of the sequence WRPF at its C terminus (Figure 2C). Interestingly, none of the 39 predicted *C. elegans* bHLH proteins, including all six members of the *hairy* subfamily, contain a GROUCHO interaction domain [19]. Thus, bHLH proteins undergo changes in their domain structure during nematode evolution.

ped-6 Encodes a *groucho*-like Gene

We mapped the *ped-6* locus by using a similar strategy. *ped-6* maps to chromosome V close to the marker S223, which is associated with the BAC clone PPBAC15-E02 of contig 66 of the physical map of *P. pacificus* (Figure 3A) [3, 4]. More accurate mapping revealed an interval of five BAC clones between the markers S231 and

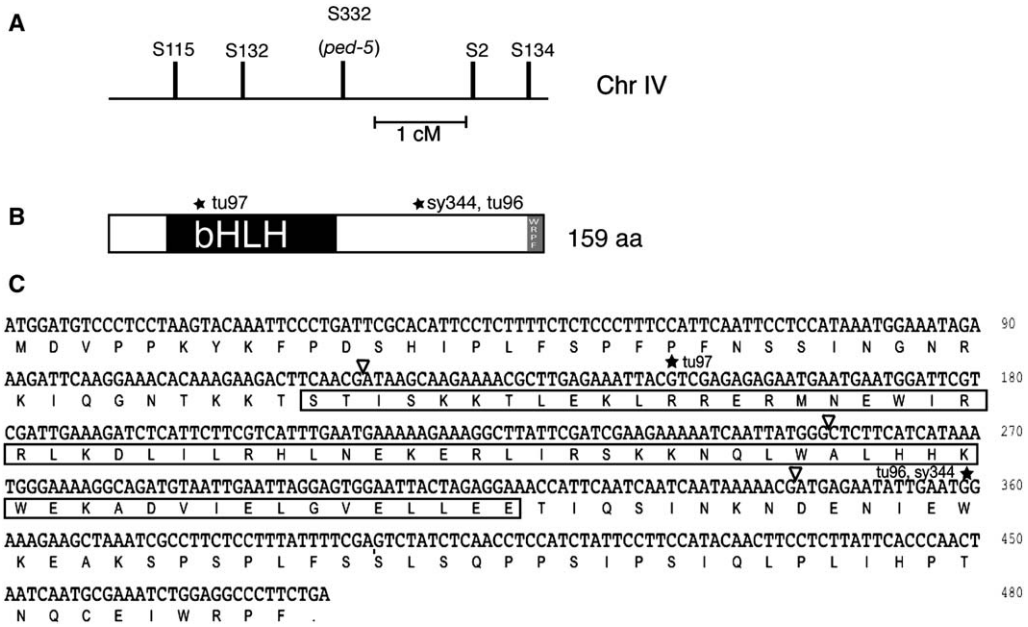

Figure 2. Molecular Cloning of *Ppa-ped-5/hairy*

Mapping has been performed with the polymorphic reference strain *P. pacificus* var. Washington [3, 4].

(A) Map position of *Ppa-ped-5/hairy* on chromosome IV between the SSCP markers S115 and S2.

(B) Protein domain structure of *Ppa*-HAIRY. *Ppa*-HAIRY has a typical bHLH domain (black box) and a GROUCHO interaction domain at its C terminus (gray box).

(C) *Ppa-hairy* cDNA sequence as obtained from 5′ and 3′ RACE experiments. Introns are indicated by open triangles. Conceptual translation starts with the first in-frame ATG codon after the SL1 splice acceptor site. Point mutations are indicated by asterisks. *tu97* is an amino acid replacement of arginine 51 to serine. In *tu96* and *sy344*, tryptophane 120 is mutated to a TGA and TAG stop codon, respectively.

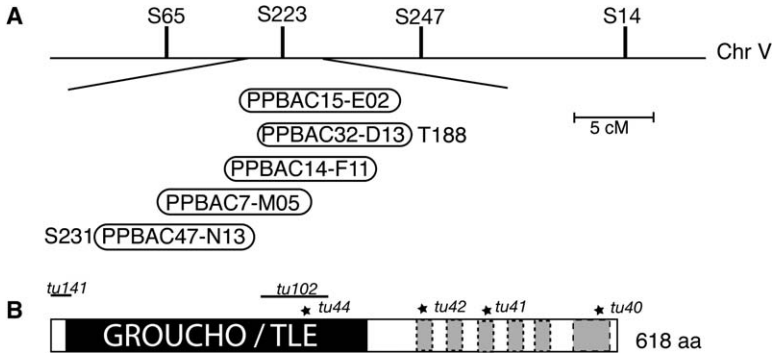

Figure 3. Molecular Cloning of *Ppa-ped-6/groucho*

Mapping has been performed as described for *Ppa-hairy*.

(A) Map position of *Ppa-ped-6/groucho* at the tip of chromosome V between the markers S231 and T188.

(B) Protein domain structure of *Ppa*-GROUCHO with a typical interaction domain (black box) and six WD repeats (gray box). Point mutations are indicated by asterisks and deletions by bars.

T188 in which *ped-6* is located. To clone the *ped-6* gene, we performed shotgun sequencing of the BAC clones between S231 and T188 and identified a sequence in the PPBAC14-F11 clone with strong similarity to the *unc-37/groucho* gene (Figures 3B, 4A, and 4B). *unc-37/groucho* encodes a nuclear protein that has been implicated as a corepressor of HAIRY and TCF/LEF-1 in many animal systems. TCF/LEF-1-like molecules are HMG box transcription factors acting downstream of Wnt signaling [20].

Next, we cloned *Ppa-groucho* as a candidate gene for the *ped-6* locus and identified mutations in all tested *ped-6* alleles (Figure 4A, Table 2). We conclude that *Ppa-groucho* is identical to *ped-6* and have renamed the gene accordingly. *Ppa*-GROUCHO is highly similar to *Cel*-UNC-37/GROUCHO with an overall amino acid sequence identity of 46% (Figure 4B). The highest amino acid similarity is found in the six WD repeats of the protein, which were shown to be involved in protein-protein interactions [21]. The variable domains in the N-terminal part of the protein are less conserved but still show significant similarity to *Cel*-UNC-37/GROUCHO.

Ppa-HAIRY and *Ppa*-GROUCHO Repress the Hox Gene *Ppa-lin-39*

Given that *Ppa-hairy* and *Ppa-groucho* have an identical phenotype with respect to P(3,4).p survival, we hypothesized that they might affect the size of the VEG by repressing, as a heterodimer, the Hox gene *Ppa-lin-39*. Several independent observations support this hypothesis. To determine whether *Ppa*-HAIRY can physically interact with GROUCHO, we used a yeast two-hybrid system. A full-length *Ppa*-HAIRY protein interacts with nematode and insect GROUCHO (Figure 5A). One potential target of the *Ppa*-HAIRY protein is the Hox gene *Ppa-lin-39*, which specifies the VEG. *Ppa-lin-39* mutants have a vulvaless phenotype because of programmed cell death of P(5–8).p (Table 1, line 6) [22]. To determine

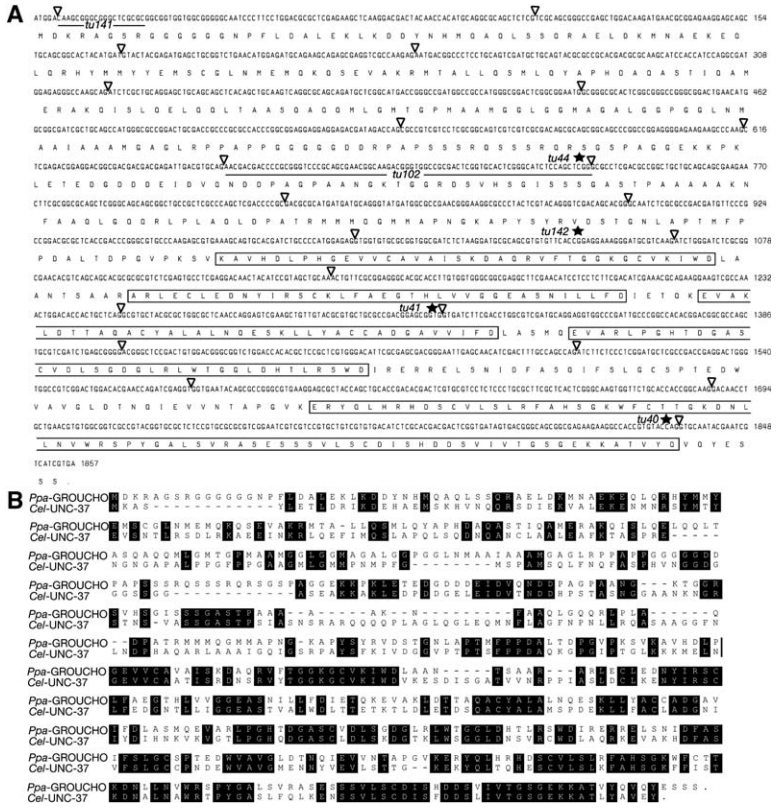

Figure 4. *P. pacificus ped-6* Gene Structure and Alignment to *C. elegans unc-37*

(A) *Ppa-groucho* cDNA sequence as obtained from 5′ and 3′ RACE experiments. Conceptual translation starts with the first in-frame ATG codon after the SL1 splice acceptor site. Introns are indicated by open triangles. Point mutations are indicated by asterisks, deletion mutations by lines. The WD repeats are boxed.

(B) Amino acid comparison between *Ppa*-GROUCHO and *Cel*-UNC-37/GROUCHO.

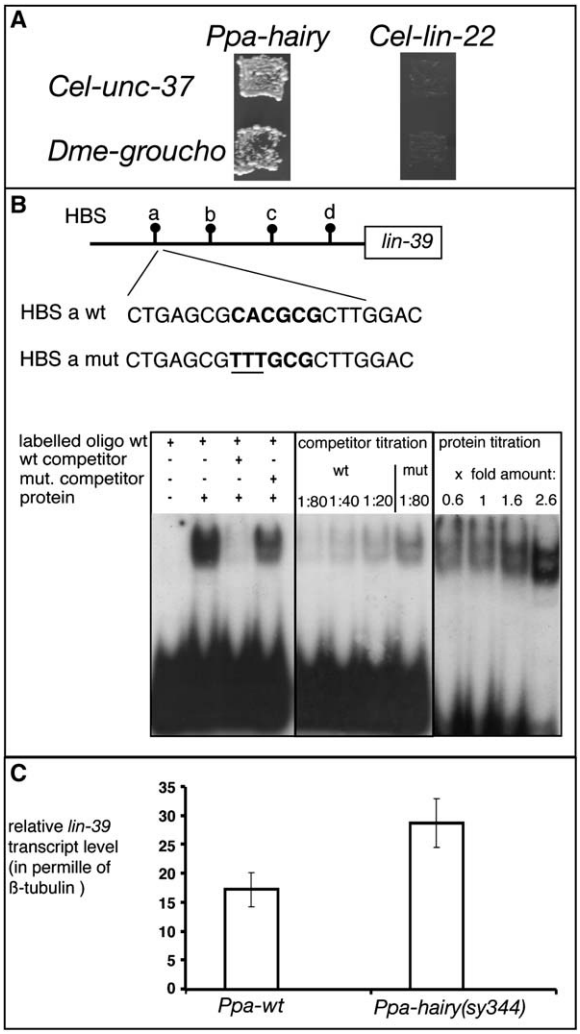

Figure 5. *Ppa*-HAIRY and *Ppa*-GROUCHO Repress the Hox Gene *Ppa-lin-39*

(A) *Ppa*-HAIRY, but not *Cel*-LIN-22, interacts with *C. elegans* or *Drosophila* GROUCHO in a yeast two-hybrid assay. *Ppa*-HAIRY and *Cel*-LIN-22 were fused to the Gal4 DNA binding domain (BD) and *Cel*-GROUCHO and *Drosophila* GROUCHO were fused to the Gal4 activation domain (AD) with the pGBKT7 and pGADT7 vectors (Clontech, Palo Alto, CA). Interactions were tested by growth assays on SD-Ade-His-Leu-Trp agar plates.

(B) Putative HAIRY binding site in the *Ppa-lin-39* promoter. FUZZNUC (http://bioweb.pasteur.fr/seqanal/interfaces/fuzznuc.html) analysis of the *Ppa-lin-39* promoter including 10 kb of upstream sequence revealed the presence of four putative HAIRY binding sites (HBS) ("a" to "d"), two of which are high-affinity binding sites. Beneath is shown the nucleotide sequence of the predicted HAIRY binding site "a" with the core hexanucleotide CACGCG. In HBS a mut, the core hexanucleotide is mutated toward TTTGCG. In electrophoretic mobility shift assays, GST-*Ppa*-HAIRY binds to oligonucleotides of the wild-type *Ppa-lin-39* sequence, but not the mutated site.

(C) *Ppa-lin-39* transcript level is upregulated in *Ppa-hairy* mutants. Transcript levels are given as arbitrary concentration unit ratios between *lin-39* and *β-tubulin* as internal standard. RNA was prepared from 120 J1 animals and experiments were carried out in duplicate. Error bars represent standard deviation.

whether *Ppa-hairy* and *Ppa-groucho* function in P(3,4).p requires *Ppa-lin-39* activity, we analyzed the respective double mutants. The anterior cells P(3,4).p underwent programmed cell death in *Ppa-hairy(sy344); Ppa-lin-39(tu29)* and *Ppa-groucho(tu102); Ppa-lin-39(tu29)* double mutants (Table 1, lines 7 and 10). The partial rescue of the cell-death phenotype in these double mutants is due to the fact that *Ppa-lin-39(tu29)* is not a null allele. We conclude that *Ppa-hairy* and *Ppa-groucho* function requires *Ppa-lin-39* activity. To explore the idea that *Ppa*-HAIRY directly regulates *Ppa-lin-39*, we searched for potential binding sites in the *Ppa-lin-39* promoter and found three putative HAIRY binding sites that conform to the sequence GGCACGYGHY (Figure 5C) [23]. We tested whether *Ppa*-HAIRY could bind these putative sequences by using electrophoretic mobility shift assays (Figure 5B). *Ppa*-HAIRY caused a shift in mobility of an oligonucleotide containing a 10 bp high-affinity binding site present in the *Ppa-lin-39* promotor but did not cause a shift in an oligonucleotide in which the binding site had been mutated. By means of an unspecific competitor in which three central base pairs had been mutated, the binding of *Ppa*-HAIRY to the oligonucleotide reoccurred, showing that the binding is specific. Finally, we tested for deregulation of *Ppa-lin-39* transcription in *Ppa-hairy(sy344)* mutants by using quantitative RT-PCR experiments (Figure 5C). In comparison to wild-type animals, *Ppa-lin-39* expression is upregulated in *Ppa-hairy(sy344)* mutants animals (Figure 5C). Synchronized *Ppa-groucho* mutants cannot be generated, given their strong egg laying-defective phenotype; quantitative RT-PCR experiments performed with mixed-staged *Ppa-groucho(tu102)* mutant animals also suggest a *Ppa-lin-39* upregulation in comparison to wild-type (data not shown). We conclude that *Ppa-hairy* and *Ppa-groucho* require *Ppa-lin-39* for the function in P(3,4).p. *Ppa*-HAIRY can form a heterodimer with GROUCHO molecules in a yeast two-hybrid system and binds to *Ppa-lin-39* promoter elements in vitro. *Ppa-lin-39* transcription is upregulated in *Ppa-hairy* and *Ppa-groucho* mutants. These experiments suggest that the anterior border of the *P. pacificus* VEG is actively restricted by *Ppa*-HAIRY- and *Ppa*-GROUCHO-mediated repression of the Hox gene *Ppa-lin-39*.

Table 3. Genetic Regulation of the Vulva Equivalence Group by *Cel-lin-22* and *Cel-mab-5*

	Genotype	P3.p 3°	%
1	Wild-type N2	16/30	53
2	*Cel-lin-22(mu2)*	30/30	100
3	*Cel-lin-22(mu2); Cel-mab-5(e1239)*	13/24	54

Cel-LIN-22 Is Not an Ortholog of *Ppa*-HAIRY and Does Not Physically Interact with GROUCHO

The experiments described above address the restriction of the VEG in the anterior body region of *P. pacificus*, but not the control of the VEG in the model organism *C. elegans*. In *C. elegans*, the cell lineage of the anterior-most cell of the VEG, P3.p, is variable within genetically homogenous populations and is polymorphic between wild isolates [15]. In the laboratory strain N2, P3.p has a 3° fate in 50% of the animals, whereas it is not part of the VEG and fuses with the hypodermis in the remaining 50% of the animals (Table 3, line 1). *Cel-lin-22* encodes a bHLH molecule of the HAIRY family that affects the anterior border of the VEG [17, 24]. P3.p

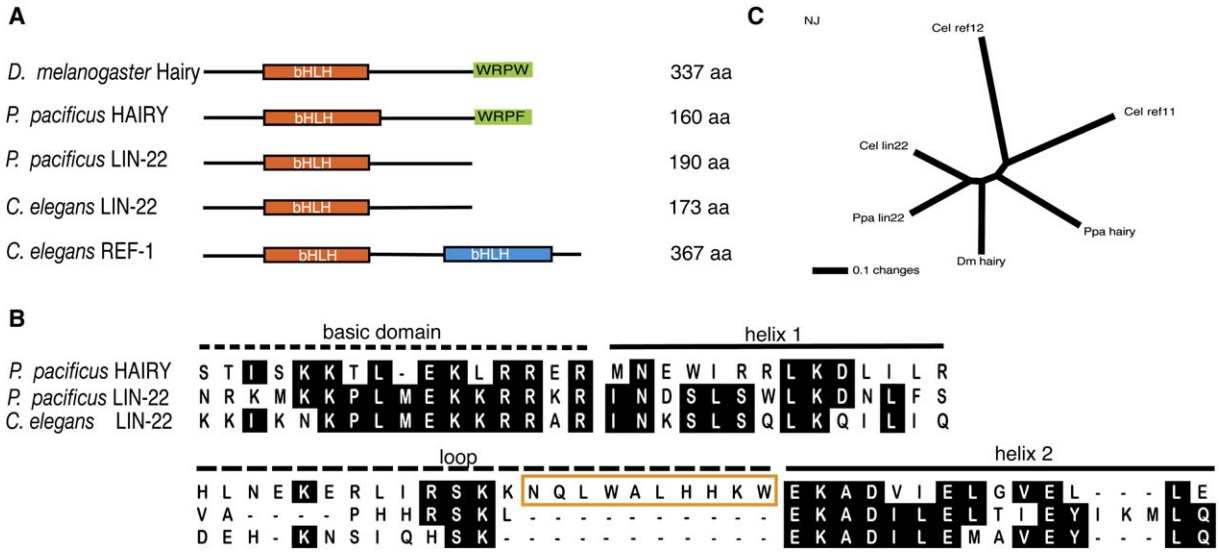

Figure 6. Structure and Function of Nematode and Insect HAIRY-like Proteins

(A) Comparison of the domain structure of HAIRY-like proteins. *Ppa*-HAIRY and *Drosophila*-HAIRY contain a bHLH (brown and red) and a GROUCHO interaction domain (green). *Cel*-LIN-22 and *Ppa*-LIN-22 both miss the GROUCHO interaction domain. Other members of the *C. elegans* REF-1 family contain two bHLH domains, a feature not known outside *Caenorhabditis* nematodes.

(B) Amino acid sequence comparison of the bHLH domain of *Ppa*-HAIRY, *Cel*-LIN-22, and *Ppa*-LIN-22. The loop region is expanded in *Ppa*-HAIRY (brown box). *Ppa*-LIN-22 and *Cel*-LIN-22 share 12 unique amino acids, whereas the two *P. pacificus* proteins share only four unique amino acids in the bHLH domain.

(C) Neighbor joining tree of the bHLH domains of *Ppa*-HAIRY, *Drosophila*-HAIRY, the *C. elegans* and *P. pacificus* LIN-22 proteins, and the two bHLH domains of *Cel*-REF-1 indicates that *Ppa*-HAIRY is not the ortholog of *Cel*-LIN-22.

is a member of the VEG in 100% of *Cel-lin-22* mutants, resulting in a slight expansion of the VEG at the population level (Table 3, line 2). Therefore, it could be assumed that *Cel-lin-22* fulfills a similar function in the specification of the VEG as *Ppa-hairy* in *P. pacificus*.

A comparison between their protein sequences reveals that *Ppa*-HAIRY and *Cel*-LIN-22 have several differences, indicating that *Cel*-LIN-22 is not the ortholog of *Ppa-hairy* (Figure 6). The *P. pacificus* genome encodes another bHLH protein that is more closely related to *Cel*-LIN-22 than *Ppa*-HAIRY, and we have designated this gene as *Ppa*-LIN-22. *Ppa*-LIN-22 and *Cel*-LIN-22 differ from *Ppa*-HAIRY and other HAIRY-like proteins in that they do not contain a GROUCHO interaction domain (Figure 6A). *Ppa*-HAIRY differs from the LIN-22 proteins by an extended loop and several unique amino acids in the bHLH domain (Figure 6B). Finally, there is no other protein encoded in the *C. elegans* genome that has a higher similarity to *Ppa*-HAIRY.

To test whether the absence of the GROUCHO interaction domain in *Cel*-LIN-22 results in the failure of the protein to interact with GROUCHO, we used the yeast two-hybrid system and carried out similar experiments as described for *Ppa*-HAIRY. Indeed, *Cel*-LIN-22 does not interact with *Drosophila* GROUCHO or *Cel*-UNC-37/GROUCHO (Figure 5A). We conclude that the short C-terminal motif is crucial for the interaction with GROUCHO and that the loss of this interaction domain in *Cel*-LIN-22 abrogates the physical interaction with GROUCHO molecules. Accordingly, *Cel-unc-37* does not have a P3.p phenotype and was shown to be dispensable for *Cel-lin-22* function [25, 26].

The *Cel-lin-22* Vulva Phenotype Requires a Different Hox Target Gene

The Hox gene *Cel-lin-39* is necessary and sufficient for the formation of the VEG in *C. elegans* and has a role that is largely similar to that of *Ppa-lin-39* [7, 8, 22]. Genetic studies did not provide any evidence that *Cel-lin-22* would regulate *Cel-lin-39*. Instead, genetic studies showed that the primary target of *Cel-lin-22* during male development is another Hox gene *mab-5*, which encodes an *Antennapedia*-like molecule [27].

To determine whether the vulva phenotype of the *Cel-lin-22* mutant could be accounted for by changes in *Cel-mab-5* expression, we studied *Cel-lin-22; Cel-mab-5* double mutants. Indeed, the expansion of the VEG as observed in *Cel-lin-22* single mutants is completely abrogated in *Cel-lin-22; Cel-mab-5* double mutants, resulting in a wild-type vulva pattern (Table 3, line 3). These results allow two major conclusions. First, the expansion of the VEG in *Cel-lin-22* depends only on the deregulation of *Cel-mab-5*. This is consistent with earlier reports of a P3.p fusion defect in animals in which *Cel-mab-5* is overexpressed under the control of a heat-shock promoter [27]. Second, *Cel-lin-22* is not directly involved in the regulation of the anterior border of the VEG in *C. elegans* N2 animals because the VEG is specified normally in the *Cel-lin-22; Cel-mab-5* double mutant. In conclusion, *Ppa-hairy* and *Cel-lin-22* are two nonorthologous nematode genes that encode bHLH proteins with different domain structures and that have different functions and different Hox target genes in vulva development.

P(3,4).p Survival in *Ppa-hairy* Does Not Require *Ppa-mab-5*

Finally, we wanted to know whether *Ppa-hairy* regulates *Ppa-mab-5* in a way similar to the one described for *Cel-lin-22* and *Cel-mab-5*. *Ppa-mab-5* mutants have been described previously and cause strong defects in the postembryonic mesodermal lineage and the ectopic vulva differentiation of the posterior epidermal cell P8.p [28, 29]. However, *Ppa-mab-5* mutants do not alter the cell-death pattern of ventral epidermal cells. *Ppa-hairy(sy344); Ppa-mab-5(tu357)* double mutants have an additive phenotype. P(3,4).p survive in all double mutant animals and the ectopic differentiation is similar to *Ppa-mab-5* single mutants (Table 1, line 8). We conclude that *Ppa*-HAIRY does not require *Ppa-mab-5* for the regulation of the cell death of P(3,4).p. This result provides additional evidence that *Ppa-hairy* and *Cel-lin-22* have different target genes and function by different molecular mechanisms.

Discussion

Our experiments provide genetic, molecular, and biochemical evidence that a complex of *Ppa*-HAIRY and *Ppa*-GROUCHO determines the size of the VEG. The proposed mechanism resembles the function of HAIRY and GROUCHO in insects and vertebrates. Although the VEG in *P. pacificus* is obviously derived, it is established by a molecular module that no longer exists in *C. elegans*. Indeed, multiple studies indicate the absence of a typical HAIRY/GROUCHO module in *C. elegans* [19]. First, *C. elegans* does not have an ortholog of *Ppa-hairy*. Although *P. pacificus* and *C. elegans* share a *lin-22* gene, *Ppa-hairy* is unique to *P. pacificus*. This is most likely the result of a complete loss of this gene in the *C. elegans* lineage. Alternatively, the *Ppa-hairy* gene might be the result of a gene duplication in the *P. pacificus* lineage. The available whole-genome sequences of other *Caenorhabditis* species do not contain *Ppa-hairy*-like genes, suggesting that the potential gene loss occurred earlier in the evolutionary lineage leading to *C. elegans*. The upcoming genome-sequencing projects of nematodes that represent more ancestral phylogenetic lineages, such as *Brugia* and *Trichinella,* might be able to shed new light on this question (http://www.nhgri.nih.gov/12511858). Second, the five additional *hairy*-like genes of *C. elegans*, the so-called *ref-1* family, are unusual. They contain two bHLH domains, a feature so far known only from *C. elegans*, and none of them contains a GROUCHO interaction domain (Figure 6A) [19]. Third, *Cel-unc-37/groucho* does not have a phenotype similar to *Cel-lin-22* and *Cel-ref-1*, the two *hairy*-like genes that result in cell-fate specification defects when mutated [25]. Fourth, none of the *hairy*-like genes in *C. elegans* is directly involved in the regulation of the VEG in the anterior body region. Although these four arguments strongly suggest the absence of a *Ppa-hairy* ortholog in *C. elegans*, rapid sequence divergence might prevent the recognition of this gene. Therefore, only a phylogenetic analysis with additional whole-genome sequence data (once available) can ultimately determine the complete absence of a *hairy* ortholog in *C. elegans*.

Experiments described in this study suggest that the vulva phenotype of *Cel-lin-22* results from the ectopic expression of *Cel-mab-5*. Ectopic *Cel*-MAB-5 expression prevents cell fusion of P3.p, a function that differs from its role in regulating cell fusion in the posterior VPCs P7.p and P8.p [30]. We favor a model in which the anterior border of the VEG in *C. elegans* depends on stochastic fluctuation of LIN-39 activity. This is supported by the observation that P3.p fusion varies strongly between natural populations of *C. elegans* [15]. Other than these natural populations, only multivulva mutations in the EGF, Wnt, and synMuvB pathways bring P3.p into the VEG [16].

This study concentrates on the cell-survival phenotype of *Ppa-groucho* that causes an extension of the VEG. In addition, *Ppa-groucho* mutants show a multivulva and gonad-independent differentiation phenotype, indicating a *Ppa-hairy*-independent role of *Ppa-groucho* as part of a negative signal that prevents vulva formation in *P. pacificus*. Previous studies revealed that vulva formation in *P. pacificus* requires two patterning aspects that strongly differ from *C. elegans*: multiple cells of the somatic gonad are involved in vulva induction in *P. pacificus*, whereas vulva induction in *C. elegans* requires only the AC [6, 31]. In addition, a negative signal in *P. pacificus* counteracts vulval induction by the somatic gonad, which is, in this form, unknown from *C. elegans*. Wnt signaling is part of this negative signaling system in *P. pacificus* [18]. The phenotype of *Ppa-groucho* mutants and the known interactions of WNT and GROUCHO in other animal systems suggest that *Ppa*-GROUCHO acts downstream of or in parallel to Wnt signaling and functions as a corepressor of the negative signal in *P. pacificus*. This function of GROUCHO is independent of the HAIRY/GROUCHO module-dependent patterning of P(3,4).p, which occurs much earlier in larval development. Future studies will reveal whether the different regulation of LIN-39 in both nematodes is connected to the different downstream functions, such as the regulation of cell fusion (*C. elegans*) versus cell death (*P. pacificus*).

Conclusion

The comparison between *P. pacificus* and *C. elegans* indicates two major differences in the patterning mechanisms underlying vulva formation. First, the HAIRY/GROUCHO module is used in *P. pacificus* to restrict the anterior border of the VEG, but is absent from *C. elegans*. Second, vulva induction and the role of Wnt signaling in this process have been substantially modified during nematode evolution [18]. Thus, changes of the inductive properties of the gonad and the competence of the responding epidermis involve the recruitment of distinct molecular modules (HAIRY/GROUCHO) and changes in gene function (LIN-17/FRIZZLED).

Experimental Procedures

Nematode Strains and Cultures

Worms were grown on 5 cm NG agar plates seeded with OP50, a uracil-requiring mutant of *E. coli* [32]. The following strains were used in this study: *P. pacificus* PS312 (the wild-type strain) is a derivative of an isolate from Pasadena, CA; *P. pacificus* PS1843 is isolated from Port Angeles, WA [33].

Cell Ablation Experiments

Animals were picked into M9 buffer placed on a pad of 5% agar in water containing 10 mM sodium azide as anesthetic. All ablation

experiments were carried out 0–1 hr after hatching of the larvae (20°C) and were carried out as described elsewhere [34].

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 hr at 20°C. The suspension was washed in M9 five times, and the worms were spotted onto the surface of NG plates. After 1 hr, excess liquid had been absorbed and individual motile J4 hermaphrodites were picked individually to plates. In the F2 generation, egg laying-defective mutants were isolated and their progeny were reanalyzed for vulva defects by Nomarski microscopy. Mutant hermaphrodites were backcrossed multiple times with wild-type males. Complementation tests were carried out with morphological markers [35].

Mapping and SSCP Detection

For mapping, mutant hermaphrodites in the California background were crossed with males of the Washington strain. To extract genomic DNA, F2 mutant animals were picked to single tubes containing 2.5 μl of lysis buffer (50 mM KCl; 10 mM Tris-HCl [pH 8.3]; 2.5 mM $MgCl_2$; 0.45% NP-40; 0.45% Tween; 0.01% gelatin; 5 μg/ml Proteinase K) and incubated for 1 hr at 65°C, followed by inactivation of the Proteinase K at 95°C for 10 min. To assign linkage of a mutation to a certain chromosome, two representative SSCP markers per chromosome were tested against 42 Washington-backcrossed mutant animals. For SSCP detection, PCR samples were diluted 1:1 in denaturing solution (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue), denatured at 95°C for 5 min, and loaded onto a GeneGel Excel prepoured 6% acrylamide gel (PharmaciaBiotech, Piscataway, NJ). Gels were fixed and silver stained to detect the DNA.

Quantitative PCR Experiments

120 J1 animals were picked into 15 μl of 1:10 diluted single worm lysis buffer. RNA was extracted with 100 μl of TRIZOL with a repeated freeze-thaw protocol in liquid nitrogen. RNA was reverse transcribed in 20 μl total volume reaction (Invitrogen) with negative controls without reverse transcriptase included for each sample. Quantitative PCR was performed on a Roche LC480 Light Cycler with the manufacturer's SYBR green PCR mix. Primer concentrations were 0.5 mM.

Electrophoretic Mobility Shift Assay

A full-length *Ppa-hairy* cDNA fragment was cloned into pGEX4-T1; GST-HAIRY protein was isolated with standard methods on a AKTAprime chromatography system (Amersham, Uppsala, Sweden). Complementary oligos were annealed and end labeled with polynucleotide kinase (PNK). 1.5 pmol of labeled oligonucleotides was used in bandshift experiments. Wild-type and mutated unlabeled oligonucleotides were used at the fold amounts indicated in competition experiments.

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