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*PNAS* published online Jun 5, 2007;

doi:10.1073/pnas.0610799104

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# Evolution of robustness in the signaling network of *Pristionchus* vulva development

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Edited by Sean Carroll, Howard Hughes Medical Institute/University of Wisconsin, Madison, WI, and accepted by the Editorial Board May 3, 2007 (received for review December 6, 2006)

**Robustness to environmental or genetic perturbation, like any other trait, is affected by evolutionary change. However, direct studies on the interplay of robustness and evolvability are limited and require experimental microevolutionary studies of developmental processes. One system in which such microevolutionary studies can be performed is vulva development in the nematode *Pristionchus pacificus*. Three vulval precursor cells respond to redundant cell–cell interactions, including signals from the gonad and the epidermal cell P8.p. Interestingly, *P. pacificus* P8.p is involved in cell fate specification of the future vulva cells by lateral inhibition but is incompetent to respond to the inductive signal from the gonad itself. These functional properties of P8.p are unknown from other nematodes, such as *Caenorhabditis elegans*. We began an experimental and genetic analysis of the microevolution of P8.p function. We show that vulva misspecification events differ between *Pristionchus* strains and species. Similarly, lateral inhibition and developmental competence of P8.p evolved within the genus *Pristionchus* and between natural isolates of *P. pacificus*. Surprisingly, in some recombinant inbred lines of two distinct *P. pacificus* isolates, P8.p gained competence to form vulva tissue, a trait that was never observed in *P. pacificus* isolates. Our results suggest differences in developmental stability between natural isolates, and we hypothesize that the remarkable evolvability of redundant cell–cell interactions allows for adaptive evolution of robustness to developmental noise.**

evolvability | developmental stability

Living organisms are remarkably stable in the face of environmental and genetic perturbation. This property, often called robustness (1) or developmental stability, reduces the amount of phenotypic variation that is visible to selection. However, organisms do evolve, and the accumulation of mutations results in changes of modular components, i.e., molecules, networks, and cells, eventually resulting in developmental and morphological novelty. Robustness itself can evolve as well. This is especially evident in the case of robustness to developmental noise: mutations that confer higher robustness in a given environment might increase in frequency by standard evolutionary mechanisms (1). For a full understanding of the evolution of robustness, research activities in evolutionary genetics and evolutionary developmental biology have to be combined. More precisely, direct studies on the interplay of robustness and evolvability, i.e., the capacity to generate heritable selectable phenotypic variation (2), require experimental microevolutionary studies of developmental processes (3).

One developmental system in which the robustness of signaling systems can be studied with single-cell resolution and by genetic and molecular analyses is the nematode vulva. In the model organism *Caenorhabditis elegans*, various cell–cell interactions are involved in vulval cell fate specification and require activities of EGF, Wnt, and lin-12/Notch signaling pathways (4). The diplogastrid nematode *Pristionchus pacificus* shares many experimental advantages with *C. elegans* but differs in several aspects of vulva development (5). Although redundant cell–cell interactions and the activity of signaling systems are also

crucial for *P. pacificus* vulva formation, the exact identity of cellular and molecular components differs strongly between the two species (5). In short, the nematode vulva is a derivative of the ventral epidermis, which consists of 12 blast cells (P1.p–P12.p) [supporting information (SI) Fig. 4]. In *P. pacificus*, four of these 12 blast cells (P5.p–P8.p) are selected to form a vulva equivalence group. Later in development, three of the four vulva precursor cells (VPCs), P(5–7).p, are induced by a signal from the gonad and adopt one of two alternative vulval fates (Fig. 1). P5.p and P7.p have the 2° fate and generate seven progeny each that build the outer part of the vulva. P6.p has the 1° fate and generates six progeny that form the inner part of the vulva. The only surviving blast cell not directly involved in vulva formation is the posterior cell P8.p (Fig. 1a). P8.p remains epidermal and is assigned a 4° fate, because it does not contribute to vulva tissue and, contrary to the homologous cell in *C. elegans*, is not competent to respond to inductive signal in the absence of other VPCs (6). Thus, the vulva equivalence group of *P. pacificus* is asymmetric in respect to the gonadal anchor cell, with a fate pattern 2°–1°–2°–4° (Fig. 1a). Interestingly, P8.p is involved in a cell–cell interaction that regulates vulva cell fate specification in a redundant manner. P8.p, together with the mesoblast M, prevents P5.p and P7.p from adopting the 1° fate in the absence of P6.p (Fig. 1b) (6). For example, if P(6,7).p are ablated, P5.p will adopt a 2° fate in the majority of cases (88%), whereas it will adopt a 1° fate if P(6,7).p are ablated together with P8.p (Fig. 1b and c). This interaction has been designated as “lateral inhibition” (LI) and is unknown from *C. elegans* and other nematodes. Because of the asymmetric configuration of the *Pristionchus* vulva equivalence group, we refer to the P8.p effect on P7.p and P5.p as short- and long-range LI, respectively. Interestingly, ablation of P8.p alone does not alter the VPCs’ lineages, indicating that LI represents a redundant signaling system that affects 2° cell fate specification of P(5,7).p, together with the inductive signal from the somatic gonad. Thus, P8.p-mediated signaling might contribute to robustness of the developmental module of the vulva.

Here we study robustness and evolvability of the developmental program of *Pristionchus* vulva development by analyzing developmental misspecification events and microevolutionary differences in P8.p-mediated signaling. In recombinant inbred lines (RILs), we investigate the evolvability of this signaling network and observe the emergence of previously undescribed characteristics in redundant aspects of cell–cell signaling. We hypothesize that this remarkable evolvability of redundant cell–

Author contributions: H.Z. and R.J.S. designed research; H.Z. performed research; H.Z. analyzed data; and H.Z. and R.J.S. wrote the paper.

The authors declare no conflict of interest.

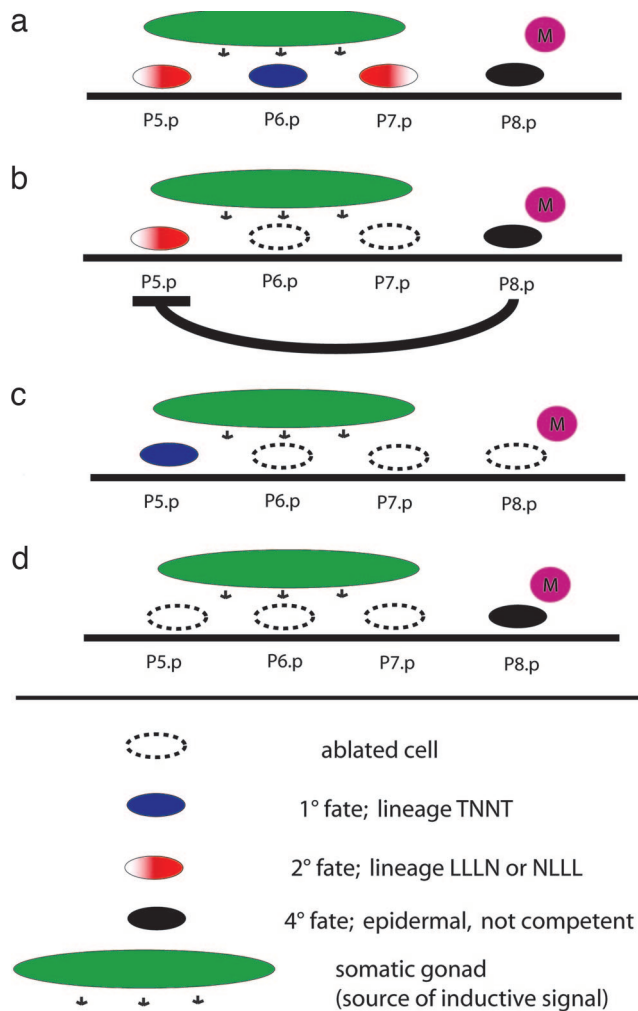
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Abbreviations: VPC, vulva precursor cell; LI, lateral inhibition; RIL, recombinant inbred line; SSCP, single-strand conformational polymorphism.

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This article contains supporting information online at [www.pnas.org/cgi/content/full/0610799104/DC1](http://www.pnas.org/cgi/content/full/0610799104/DC1).

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**Fig. 1.** P8.p is the source of an inhibitory signal. (a) P(5–7).p are induced to give rise to vulva tissue by the somatic gonad; P8.p remains epidermal. P5.p and P7.p have the 2° fate (seven progeny) and form the outer part of the vulva. P6.p has the 1° fate (six progeny) and forms the central part of the vulva. (b) P5.p adopts the 2° fate after ablation of P(6,7).p. (c) If P8.p is ablated together with P(6,7).p, P5.p adopts the 1° fate, indicating that P8.p is the source of an inhibitory signal. The same logic applies to ablation of P(5,6).p and P(5,6,8).p, respectively. (d) P8.p itself cannot respond to gonadal signaling, even in the absence of all other VPCs. L, N, and T refer to cell division patterns in the last round of VPC division: L, longitudinal; N, nondividing; T, transversal.

cell interactions allows for adaptive evolution of robustness to developmental noise.

## Results

***Pristionchus* Vulva Cell Fate Specification Is Robust.** To study to what extent *Pristionchus* vulva development is affected by developmental noise and to investigate what kind of developmental errors can occur in different genetic backgrounds, we analyzed VPC specification patterns in >4,000 worms belonging to two strains of *P. pacificus* and two strains of *Pristionchus entomophagus* (Table 1). In all strains, the developmental program is executed with high precision; only between 0.2% and 1.1% of all examined animals showed VPC misspecifications. In *P. pacificus* PS1843, vulva-centering mistakes are prevalent: P7.p instead of P6.p had the 1° fate in six of 1,039 PS1843 animals. Very rarely, we also observed that P7.p adopted a 3° fate or was not present. This is in stark contrast to what we find in *P. entomophagus*: in strain RS145, by far the most common error is a misspecification

of P5.p or P7.p, which normally have the 2° fate. In 10 of 1,134 animals (0.9%), P5.p or P7.p formed an independent invagination (“D” fate in Table 1; Fig. 2).

Interestingly, there are strain-specific differences in the frequency at which any type of misspecification is observed. *P. entomophagus* RS144 and *P. pacificus* RS106 both show few “errors” compared with their conspecific strains RS145 and PS1843, respectively (Table 1). To test whether *P. pacificus* vulva development is affected by genetic perturbation, we determined the frequency of vulva misspecifications in a hybrid genetic background. We analyzed VPC specification patterns in 503 F<sub>2</sub> animals from a cross between RS106 and PS1843 and found only four misspecification events (0.8%), which is in the same range as in PS1843 animals (Table 1). Taken together, the *Pristionchus* vulva is a developmental module that is robust to developmental noise, and misspecification events are in the promille range only. However, the observed misspecification events indicate differences in the exact types of errors in different strains and species. This result might indicate differences in cell–cell signaling pathways that contribute to developmental stability in the respective strains and species.

## Cell–Cell Signaling Systems Vary Between *Pristionchus* Strains and Species.

To determine whether the cell–cell signaling systems involved in *Pristionchus* vulva development undergo microevolutionary changes, we analyzed short- and long-range LI in 489 ablated worms belonging to eight strains of *P. pacificus* from North America, Asia, and Europe and nine strains of the three closely related hermaphroditic species *Pristionchus maupasi*, *P. entomophagus*, and *Pristionchus* sp. 6. The vulva cell fates of unablated animals in all examined strains and species show the typical 2°–1°–2°–4° pattern as described for the *P. pacificus* laboratory strain PS312 from California (data not shown). In *P. pacificus* PS312, the presence of P8.p strongly inhibits P5.p from adopting the 1° fate after P(6,7).p ablation (6) (Fig. 1). To test whether there is natural variation for this trait, we carried out P(6,7).p ablation experiments in 17 *Pristionchus* strains (Fig. 3, black bars; SI Table 4). Compared with *P. pacificus*, P5.p has the 1° fate more often in *P. maupasi* (63% of the animals, averaged over the strains;  $P \ll 0.0001$ ), *P. entomophagus* (74%;  $P \ll 0.0001$ ) and *Pristionchus* sp. 6 (50%;  $P < 0.05$ ) (Fig. 3; SI Table 4). At the intraspecific level, seven of the eight *P. pacificus* strains show LI that is not significantly different from PS312 (Fig. 3; SI Table 4). However, in *P. pacificus* RS106 from Poland P5.p had the 1° fate in 43% of ablated animals ( $P < 0.05$  for the pairwise comparison with PS312). Thus, the influence of long-range LI is reduced in this strain.

Next, we assessed the influence of short-range LI by ablating P(5,6).p and following the cell fate of P7.p (Fig. 3, gray bars). In 16 of 17 strains, P7.p has the 1° fate less often than P5.p in the equivalent ablation experiment, indicating either a stronger influence of P8.p on its neighboring VPC or intrinsic P8.p-independent differences between P5.p and P7.p. On average, P7.p had the 1° fate in 40% of P(5,6).p ablated animals in *P. entomophagus*, but only in 5–11% of the animals in *P. pacificus* ( $P \ll 0.0001$ ), *P. maupasi* ( $P < 0.01$ ), and *P. sp. 6* ( $P < 0.05$ ) (Fig. 3; SI Table 4). Thus, the influence of short-range LI is significantly reduced in *P. entomophagus*. Taken together, although the VPC fate pattern in unperturbed animals is identical in all four *Pristionchus* species, cell ablation experiments reveal differences in cell–cell signaling between species and strains. In particular, long- and short-range LI can be uncoupled over microevolutionary time: in seven of eight *P. pacificus* strains, both, P5.p and P7.p have the 1° fate only rarely in the respective ablation experiments. In contrast, the fate patterns of these two cells differ strongly if the same experiments are performed in *P. maupasi*, *P. sp. 6*, and also in the strain RS106 of *P. pacificus*.

**Table 1. Developmental noise in four *Pristionchus* strains**

	(P4.p)	P5.p	P6.p	P7.p	P8.p	RS106, <i>n</i> = 1,049	PS1843, <i>n</i> = 1,039	RS145, <i>n</i> = 1,147	RS144, <i>n</i> = 1,010	F2 PxW, <i>n</i> = 503
Wild type		2°	1°	2°	4°	1,047	1,030	1,134	1,006	499
2° specification		D	1°	2°	4	0	0	9	1	0
		2°	1°	D	4°	0	0	1	0	0
Centering		3°	D	1°	4°	0	2	0	0	0
		3°	2°	1°	4°	0	3	0	1	0
		3°	2°	1°	2°	0	1	0	0	0
		1°	2°	3°	4°	0	0	1	0	0
2° competence		2°	1°	3°	4°	0	2	0	0	3
		3°	1°	2°	4°	0	0	0	0	1
		2.5°	1°	2°	4°	1	0	0	0	0
		2°	1°	2.5°	4°	0	0	0	1	0
Cell survival		2°	1°	X	4°	0	1	0	0	0
		X	1°	2°	4°	1	0	1	0	0
		X	1°	3°	4°	0	0	0	1	0
	D	2°	1°	2°	4°	0	0	1	0	0

RS106 and PS1843 are strains of *P. pacificus*, and RS144 and RS145 are strains of *P. entomophagus*. The last column shows data from randomly picked F<sub>2</sub> animals of a cross between RS106 from Poland and PS1843 from Washington. See ref. 6 for cell fate definitions.

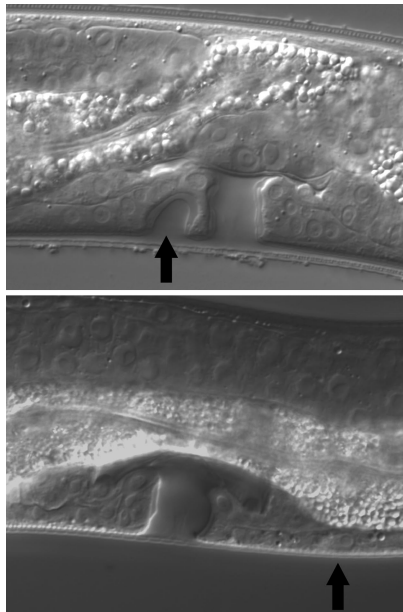
**Long-Range LI Is Lost in Some *P. pacificus* RILs.** To determine the genetic contribution to the difference in LI between *P. pacificus* strains, we analyzed F<sub>1</sub> and F<sub>2</sub> animals from crosses between RS106 from Poland and PS1843 from Washington, two strains that differ in the strength of LI ( $P < 0.05$ ) and are genetically highly polymorphic (7) (Table 2, C and D). When we crossed RS106 (weak LI) and PS1843 (strong LI) and ablated P(6,7).p in the resulting F<sub>1</sub> generation, P5.p had the 1° fate in only three of 27 animals, indicating that LI is strong in heterozygous animals ( $P < 0.01$  for the pairwise comparison with RS106). Furthermore, in randomly chosen F<sub>2</sub> animals, five of 16 P5.p cells adopted the 1° fate (31%), which is intermediate between the values found in the parental strains but not significantly different from either of them ( $P > 0.5$  for both pairwise comparisons).

To study the strength of LI in hybrid genetic backgrounds, we generated RILs of the strains RS106 and PS1843 (SI Fig. 5). We generated 16 RILs that were inbred until F<sub>13</sub> and processed them for P(6,7).p ablation. As expected, most RILs showed a pattern of P5.p fate specification that is intermediate to (38% of the RILs) or reminiscent to the parental strains (56%) (Table 2, E–T).

Surprisingly, one of the 16 RILs showed strong transgression, i.e., the occurrence of a more extreme phenotype than in either parental line (8). Specifically, P5.p had a 1° fate in >90% of the animals after ablation of P(6,7).p in RIL19C (Table 2, T;  $P < 0.001$  for the pairwise comparison with RS106; at  $P < 0.05$ , significant after Bonferroni correction for 16 pairwise comparisons). Such a pattern is not known from the two parental lines, F<sub>1</sub> hybrids thereof, or any other wild isolate of *P. pacificus*. We therefore screened another set of 58 RILs (inbred until F<sub>9</sub>) specifically for the absence of LI (SI Table 5). We found two more lines in which P5.p had the 1° fate in ≈90% of P(6,7).p-ablated animals (Table 2, U and V;  $P < 0.01$  and  $P < 0.001$  for the respective pairwise comparisons with RS106; in the case of RILA8 significant at  $P < 0.05$  after Bonferroni correction).

We selected the RILA8 as the parental strain for raising a second set of RILs (SI Fig 5). RILA8 was crossed to RS106, and the resulting second RILs were inbred until the F<sub>9</sub> generation. We expected to find RILs that lack LI at a higher ratio than in the first RILs, because RS106-derived factors that are required for the transgression phenomenon are fixed throughout the experiment. Indeed, three of 15 (20%) second RILs lacked LI, in contrast to 5% in the first RILs (Table 2, W, X, and Y; SI Table 6). The proportion of RILs that lack LI is probably an underestimate because of the possibility of false negatives in the screening procedure. We conclude that the recombination of genetic variation in RILs results in a change of cell–cell interaction patterns.

**Some RILs Gained Competence of P8.p.** To ask whether other properties of P8.p are also affected in these RILs, we further characterized the RILs that showed transgression with respect to LI. One of the intriguing properties of P8.p is that, unlike the homologous cell in *C. elegans*, it is not competent to respond to inductive signaling in the absence of P(5–7).p. By ablating P(5–7).p, we found that in RILA8 and derivatives thereof (Table 3, I–L): P8.p is competent to differentiate and form vulva tissue, which is in contrast to all tested wild isolates of *P. pacificus*



**Fig. 2.** Developmental noise in *P. entomophagus* and *P. pacificus*. (Upper) In *P. entomophagus* RS145, the most common developmental error is a misspecification of P5.p. Instead of adopting the 2° fate, P5.p forms an independent invagination (arrow) that does not connect to the uterus. (Lower) In *P. pacificus*, very rarely P7.p, instead of adopting the 2° fate, remains undivided (3° fate, arrow).



Table 2. Fate of P5.p after ablation of P(6,7).p

Strain	P5.p 1° (n)	Percent P5.p 1°	90% CI, %
A, PS1843 (Wash)	7 (33)	21	10–33
B, RS106 (Pol)	22 (51)	43	32–55
C, Pol × Wash F <sub>1</sub>	3 (27)	11	1–21
D, Pol × Wash F <sub>2</sub>	5 (16)	31	12–50
E, RIL 12B	0 (12)	0	–
F, RIL 14B	0 (6)	0	–
G, RIL 10A	2 (19)	11	0–22
H, RIL 18C	2 (11)	18	0–37
I, RIL 9A	3 (13)	23	4–42
J, RIL 2A	4 (13)	31	10–52
K, RIL 16C	2 (6)	33	2–65
L, RIL 3A	5 (14)	36	15–57
M, RIL 13B	5 (14)	36	15–57
N, RIL 25B	5 (12)	42	18–65
O, RIL 1A	5 (11)	45	21–70
P, RIL 11B	5 (11)	45	21–70
Q, RIL 17C	5 (10)	50	24–76
R, RIL 4A	4 (8)	50	21–79
S, RIL 20C	7 (11)	64	40–87
T, RIL 19C	13 (14)	93	82–100
Average (16 RILs)	4.2 (11.6)	36	
U, RIL C22	12 (13)	92	80–100
V, RIL A8	16 (18)	89	77–100
W, RIL A8BC7	19 (23)	83	70–96
X, RIL A8BC10	15 (17)	88	75–100
Y, RIL A8BC18	11 (12)	92	79–100

P(6,7).p were ablated 0–1 h after hatching, and the cell lineage of P5.p was analyzed as described in ref. 6. A, B, homozygous and isogenic laboratory strains. C, F<sub>1</sub> animals. D, segregating F<sub>2</sub> population. E–V, homozygous recombinant inbred lines. W–Y, Second-generation RILs. See text for details. Wash, Washington; Pol, Poland. CI, confidence interval.

of vulva morphology and cell lineage over evolutionary time, a general phenomenon in many nematode genera, can be attributed to robustness of developmental modules to mutation (1). Thus, constancy of the phenotype at a wide phylogenetic range contrasts with genetic variation of redundant aspects of cell–cell signaling at a microevolutionary scale.

**Correlation of LI and Robustness to Developmental Noise.** Is the natural variation in P8.p-mediated LI the outcome of neutral evolution, or is there any conceivable selection pressure that could have favored the emergence of the observed differences between closely related *Pristionchus* species? We think that changes in P8.p-mediated signaling might influence the robustness of vulva development to developmental noise. Under standard laboratory conditions, misspecifications that affect specifically the 2° fate are of opposite nature in PS1843 and RS145: whereas in PS1843 the only 2°-specific defect is a rare noninduction, in RS145, the most common defect is an ectopic differentiation. Thus, the type of P5.p/P7.p-related misspecification events correlates with the strength of LI in these strains. Although it is not yet clear whether there is a direct link between LI and robustness, our comparison of misspecification events in *Pristionchus* isolates might be interpreted as the first indication that differences in redundant cell–cell signaling affect developmental stability of *Pristionchus* vulva development. These differences might reflect adaptations that assure a robust VPC specification in the respective species-specific habitats. Future studies on the exact natural habitats of these nematodes might indicate whether developmental noise changes in different environments.

Table 3. P8.p competence in laboratory strains and RILs

Strain	P8.p D (n)	Percent P8.p D
A, PS1843	0 (21)	0
B, RS106	0 (12)	0
C, PS312*	0 (22)	0
D, JU482	0 (21)	0
E, SB5880	0 (7)	0
F, JU150	0 (6)	0
G, RIL 19C		0
H, RIL C22	0 (10)	0
I, RIL A8	7 (14)	52
J, RIL A8BC7	5 (12)	42
K, RIL A8BC10	2 (10)	20
L, RIL A8BC18	3 (4)	75
M, RIL A8BC7BC30	9 (14)	64
N, RIL A8BC7BC77	4 (8)	50
O, RIL A8BC7BC59	7 (16)	44
P, RIL A8BC7BC56	2 (7)	29
Q, RIL A8BC7BC10	3 (13)	23
R, RIL A8BC7BC47		13
S, RIL A8BC7BC20	1 (2)	50

A–F, homozygous and isogenic laboratory strains; G–S, recombinant inbred lines; G–I, J–L, and M–S are first, second, and third generations, respectively. See text for details. \*, data for PS312 from ref. 6.

**Evolvability at the Level of Redundant Cell–Cell Signaling.** In our experiments with RILs, we exemplified how redundant VPC specification signals can evolve: although vulva development is robust to genetic perturbation, at the level of redundant signaling, we observe not only transgression (loss of LI) but also novel properties (competence of P8.p). Interestingly, the three aspects of cell–cell signaling studied here in depth, long- and short-range LI and P8.p competence, can be uncoupled experimentally and also can have been uncoupled in nature. Thus, a mechanism exists in *Pristionchus* that would allow a diversification of redundant signaling events without affecting the phenotypic output (the 2°-1°-2°-4° fate pattern) in most of the animals.

**Species-Specific Characteristics of Developmental Noise.** It is illuminating to compare developmental stability in different nematode genera. The most prominent influence of developmental noise in the most-used laboratory strain of *C. elegans*, N2, concerns the fate of P3.p: in ≈50% of the animals; this cell divides once and adopts the 3° fate (4). Alternatively, it remains undivided and fuses with the epidermis during the L2 stage. The frequency of P3.p divisions varies in different strains of *C. elegans* from 15% to 59% (15). Moreover, P3.p is part of the vulva equivalence group in *C. elegans* but not in *C. briggsae*. Similar strain-specific differences in the division patterns of nonvulval cells were observed in the case of P4.p and P8.p in nematodes of the genus *Oscheius* (15). However, the 3° cell lineages do not contribute to vulva formation, and differences are not likely to be strongly selected against (15). In contrast, developmental errors in P(5–7).p, the cells that form the vulva in *Oscheius*, *Caenorhabditis*, and *Pristionchus* are rare in all three genera. In *Oscheius*, the most frequent developmental error is the absence of some Pn.p cells (≈2%), which is in contrast to the results we obtained for *Pristionchus* strains, where missing Pn.p cells are very rarely

