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Plasticity and Errors of a Robust Developmental System in Different Environments

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SUMMARY

Many developmental processes generate invariant phenotypes in a wide range of ecological conditions. Such robustness to environmental variation is a fundamental biological property, yet its extent, limits, and adaptive significance have rarely been assessed empirically. Here we tested how environmental variation affects vulval formation in *Caenorhabditis* nematodes. In different environments, a correct vulval pattern develops with high precision, but rare deviant patterns reveal the system's limits and how its mechanisms respond to environmental challenges. Key features of the apparent robustness are functional redundancy among vulval precursor cells and tolerance to quantitative variation in Ras, Notch, and Wnt pathway activities. The observed environmental responses and precision of vulval patterning vary within and between *Caenorhabditis* species. These results highlight the complex response of developmental systems to the environment and illustrate how a robust and invariant phenotype may result through cellular and molecular processes that are highly plastic—across environments and evolution.

INTRODUCTION

Robustness is a characteristic of many developmental processes that generate invariant phenotypic outputs in the presence of stochastic, environmental, and genetic variation. The mechanistic underpinnings and evolutionary significance of such developmental robustness are of long-standing and interdisciplinary interest (Flatt, 2005; Waddington, 1942; Wagner, 2005). Theoretical and experimental studies have started to uncover how developmental system properties contribute to robustness (e.g., Eldar et al., 2003; Houchmandzadeh et al., 2002; Lucchetta et al., 2005; von Dassow et al., 2000). These examples show that developmental patterning processes can tolerate considerable fluctuations in parameters defining the corresponding system. Despite these recent advances, several critical aspects concerning the robustness of developmental systems remain experimentally unresolved. First, robustness is often invoked to be a generic system property. However, the actual degree of robustness to relevant sources of variation is usually not quantified. Specifically, we lack experimental studies measuring how seemingly robust developmental systems respond to environmental variation. This is especially important because changes in the environment are believed to be the main source of developmental perturbations and the supposed primary agent favoring the evolution of robustness (Meiklejohn and Hartl, 2002). Second, rare deviations and defects in the output of a robust developmental system are generally ignored. However, such "errors" may reflect how perturbations challenge the developmental system, thereby revealing the significance of mechanistic features contributing to its robustness. Third, robustness is often regarded as an adaptive system feature without considering adaptive and nonadaptive processes underlying developmental system evolution (Lynch, 2007). Most importantly, we rarely know how robustness of a given phenotype relates to individual fitness and whether selection is required to maintain such robustness. We have aimed to address these questions by combining different experimental approaches to study a molecularly well-characterized developmental system—vulval cell fate patterning of *Caenorhabditis* nematodes—in different, ecologically relevant environments.

The vulva of the *Caenorhabditis elegans* hermaphrodite develops from a subset of ventral epidermal blast cells, the Pn.p cells (Sternberg, 2005) (Figures 1A–1C). These six cells, termed P3.p to P8.p, represent the precursor cells competent to adopt a vulval cell fate. The six cells adopt alternative cell fates. Three of them, P5.p to P7.p, adopt a vulval fate: P6.p adopts the central 1° fate, and P5.p and P7.p the outer 2° fate. P3.p, P4.p, and P8.p adopt a nonvulval fate (3° or F fate). This canonical vulval cell fate pattern is invariant within the *Caenorhabditis* genus (Félix, 2007).

The *C. elegans* vulval patterning process relies on a well-understood molecular network of intercellular signaling events. A LIN-3/epidermal growth factor (EGF) signal emanating from a specialized cell of the somatic gonad, the anchor cell, induces proximal Pn.p cells to adopt vulval cell fates via an EGF receptor (EGFR)/Ras/mitogen-activated protein kinase (MAPK) cascade. P6.p, the cell closest to the anchor cell, receives the highest dose of LIN-3/EGF, causing it to adopt the 1° fate. A lateral signal from P6.p—acting through a LIN-12/Notch pathway—induces P5.p and P7.p to adopt the 2° fate and prevents them from adopting the 1° fate by inhibiting the Ras pathway (Yoo et al., 2004). Furthermore, a lower dose of LIN-3/EGF may also induce the 2° fate in P5.p and P7.p (Katz et al., 1995). P3.p, P4.p and P8.p adopt nonvulval fates as they receive insufficient levels of either signal. In addition to Ras and Notch signaling, a canonical Wnt pathway may act to induce vulval fates, because

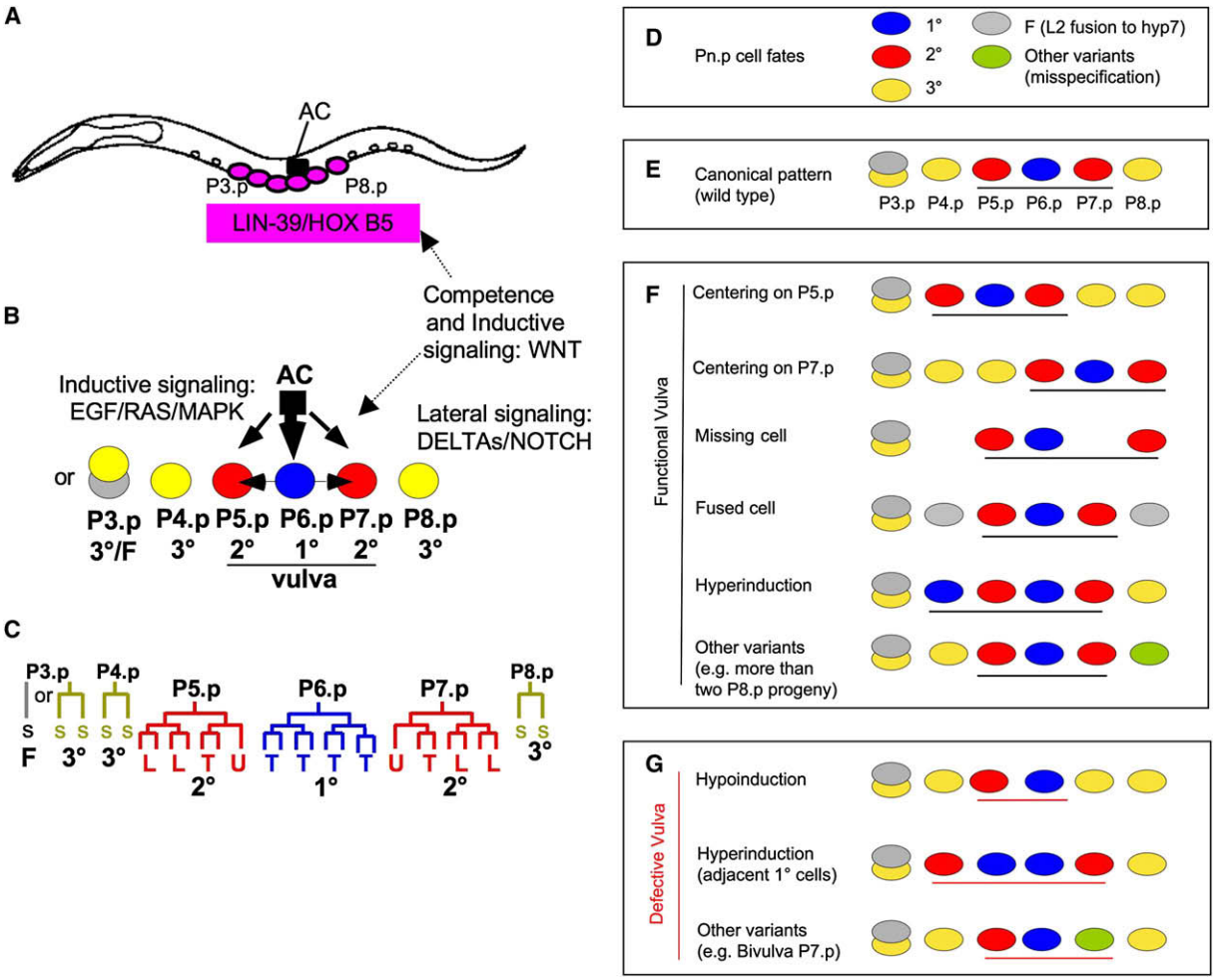

Figure 1. *Caenorhabditis* Vulval Cell Fate Patterning and Deviant Patterns in Different Environments

(A–C) Overview of *C. elegans* vulval cell fate patterning. Cells adopting vulval fates are underlined. AC, anchor cell; L, longitudinal (antero-posterior) division; S, fusion to the syncytium in the L2 stage with no division (F fate); SS, fusion to the epidermal syncytium (hyp7) after single division (3° fate); T, transverse (left-right) division; U, undivided. (A) L1–L2 stages: competence establishment and maintenance of the vulval precursor cells, P3.p–P8.p, through the expression of the Hox gene *lin-39* (pink). (B) Early-L3 stage: specification of the different vulval precursor cell fates: 1° fate (blue), 2° fate (red), 3° fate (yellow), F fate (grey). (C) Late-L3 stage: cell lineages. Each vulval fate corresponds to an invariant cell division pattern executed during the late-L3 stage, resulting in a total of 22 vulval cells. Vulval morphogenesis takes place during the L4 stage, and the complete vulval organ develops by the final moult to the adult.

(D–G) Canonical and deviant vulval cell fate patterns. See Figure S2 for images of wild-type and deviant vulval patterns in the L4 stage. (D) Possible cell fate patterns adopted by vulval precursor cells. Different fates lead to different cell lineages in the L2–L3 stages. (E) Canonical (wild-type) vulval cell fate pattern. P3.p shows stochastic behavior (Delattre and Félix, 2001) and may either fuse early to the epidermal syncytium (F fate) or divide once in the L3 stage (3° fate). (F) Variant patterns that deviate from the canonical pattern, but allow establishment of functional vulva with a complete 2°-1°-2° pattern. (G) Strongly disrupted deviant patterns leading to a nonfunctional vulva.

hyperactivation of this pathway is sufficient for vulval induction when the Ras pathway is compromised (Gleason et al., 2002).

A correct fate pattern of three vulval precursor cells (2°-1°-2°) is a prerequisite to form a functional vulva, which is essential for egg laying and mating with males. Consequently, deviations from the correct pattern can cause negative fitness effects by decreasing offspring number (see Supplemental Data available online, section 2.1) or, potentially, by impeding outcrossing. Too few induced Pn.p cells often result in a nonfunctional vulva and a decrease in offspring number. In addition, an excess of Ras signaling or defective Notch signaling may result in two adjacent 1° cells, which may cause death of the worm due to eversion of vulval tissue (Greenwald et al., 1983). The outcome of the vulval cell fate patterning process is thus linked to the reproductive success of an individual.

Caenorhabditis vulval cell fate patterning represents an ideal study system, because the vulval phenotype is a fitness-relevant trait, and because the mechanisms regulating this process are very well understood and relatively simple: fate patterning relies primarily on two signaling pathways, with the anchor cell inducing the 1° fate in P6.p (Ras pathway), which in turn induces the 2° fate in its neighboring cells, P5.p and P7.p (Notch pathway). Moreover, the vulval signaling network shows many system properties, such as positive feedback loops, switch-like behaviors, partial redundancy, and crosstalk among signaling pathways, which have been proposed to enhance its robustness

(Giurumescu et al., 2006; Kenyon, 1995; Sternberg, 2005). However, it is not known whether such developmental system properties are relevant to maintain robustness when exposed to ecologically relevant variation. Specifically, we do not know the degree of robustness and which system properties may ensure an invariant cell fate pattern when development is actually exposed to environmental perturbations. We have therefore followed *Caenorhabditis* vulval cell fate patterning in multiple environments to ask the following questions: how precise is the output of the cell fate patterning process and which errors occur in different environments? How does the environment influence the interplay and activity of molecular pathways of the vulval signaling network? Does precision of vulval patterning show evolutionary variation? What is the evidence that the precision of this process is maintained by natural selection?

RESULTS AND DISCUSSION

Precision and Errors of Vulval Cell Fate Patterns in Different Environments

We first characterized how different environments modulate the precision of the vulval cell fate patterning process. We defined six different laboratory environments covering a range of environmental stimuli aimed to mimic the type of variation likely to occur in the natural habitat of *Caenorhabditis* nematodes (Braendle et al., 2008): standard agar plates (i.e., Petri dishes containing nematode growth medium [NGM]) at 15°C, 20°C, and 25°C; liquid culture at 20°C; NGM plates with a 48 hr starvation period during the early L2 stage at 20°C; dauer pheromone plates, inducing the development of the dauer larva at 25°C (see Supplemental Data, section 1.2, available online; Figure S1). We first quantified the frequencies of canonical (F/3°-3°-2°-1°-2°-3°) versus deviant vulval cell fate patterns (Figures 1E–1G) in a single isogenic *C. elegans* isolate, N2, the reference wild-type strain. Across several experimental blocks, we scored the vulval phenotype of 1000 individuals in each of the six environments. Almost all individuals (97.65%; n = 6000) developed a canonical cell fate pattern. The frequency of strongly disrupted vulval patterns, resulting in an incomplete and likely nonfunctional vulva, was overall low (0.25%) (Figure 2A). At low frequency (2.10%), we also detected developmental deviants with noncanonical, yet complete (2°-1°-2°) vulva patterns (Figures 2B–2G). The combined proportion of all developmental deviations (Figures 1F and 1G) varied significantly among environments (generalized linear model [GLM], assuming a binomial response variable and a logit link function, main effect environment: df = 5; $\chi^2 = 32.57$; $p < 0.0001$), controlling for the effect of experimental block (GLM, interaction effect environment × block: df = 40; $\chi^2 = 73.97$; p = 0.0009; main effect block: df = 8, $\chi^2 = 6.78$, p = 0.56).

Starvation triggered an environment-specific developmental deviation: animals showed an increased propensity to center their vulva on P5.p instead of P6.p (Figure 2B). Such animals had a functional vulva (see Supplemental Data, section 2.4), with P5.p adopting a 1° and P4.p and P6.p adopting a 2° vulval fate (the anchor cell being attached to P5.p progeny). In several environments, we also found individuals missing precursor cells (Figure 2E). During development of these individuals, a functional vulva pattern could be produced due to the presence of additional precursor cells (P3.p, P4.p, P8.p) competent to adopt vulval fates. The occurrence of several developmental deviations that were tolerated and compatible with the formation of a functional vulva (Figure 1F) highlights cellular redundancy as a relevant and frequent buffering mechanism, allowing for a correct phenotypic output when exposed to environmental variation.

We found remarkably few pattern defects of the type that would be indicative of a failure in the two main components of the vulval signaling network (i.e., Ras and Notch pathways). Specifically, we never observed adjacent 1° cells despite variation in the alignment process of the 1°-fated cell and the anchor cell, indicating that lateral inhibition via the delta-Notch pathway (Sternberg, 2005) was highly efficient. The *output* of the vulva fate patterning system was therefore not easily altered or impaired by environmental variation.

Environmental Sensitivity of the Vulval Signaling Network

To analyze how the environment affects the cell fate patterning *process* underlying vulval formation, we sensitized the system using mutations in the known genetic network. Previous studies reported that the penetrance of certain vulval mutations is modified by changes in laboratory conditions (starvation, passage through dauer stage, liquid culture) (Battu et al., 2003; Ferguson and Horvitz, 1985; Moghal et al., 2003), indicating that vulval development is environmentally sensitive. Here we systematically assayed each of 41 single or double mutants derived from the N2 isolate in the six environments (Table S1; Supplemental Data, section 1.4). We found that 26/41 mutant strains showed significant variation among environments in the mean number of induced vulval cells (Figures S3–S8; Table S2; Supplemental Data, section 2.2). Such environmental effects resulted in both aggravation and alleviation of mutant phenotypes. The most frequent and consistent environmental effects were observed in starvation and dauer-inducing environments, with many reduction-of-function mutations showing elevated levels of vulval induction (Figures S3–S8).

In one striking example, hypomorphic mutations in *lin-3/egf* and *let-23/egfr*, which otherwise result in a highly penetrant, hypoinduced phenotype, were strongly suppressed in the starvation and/or dauer-inducing environments (Figures 3B; Figures S3 and S4A). Significantly, hypomorphic mutations in other Ras pathway components did not show such suppression, indicating that this environmental effect may act upstream or at the level of the EGF receptor. In addition, we also observed suppression of hypoinduction caused by mutations in downstream effectors of vulval induction, such as *lin-39* (suppressed in the dauer environment [Figure S8A]) and *sur-2* (suppressed in the starvation environment [Figure S8B]). The levels of inductive signaling activity therefore change with the environment, inducing strong alterations of the vulval phenotype in mutant but not wild-type backgrounds.

In contrast to the suppression effects observed for *lin-3/egf* or *let-23/egfr* mutations, a null mutation in the Wnt-regulated *bar-1/β-catenin*, which causes only mild hypoinduction at 20°C, was significantly aggravated in the 15°C and starvation environments (Figure 3B). This and additional results for other mutants of the Wnt cascade (Figure S7) implied that the inductive role of Wnt signaling was elevated at 15°C and in the starvation environment. As Ras and Wnt pathways have a partially redundant role

in the induction of vulval fates, these two pathways may differentially contribute to vulval induction depending on the environment—a scenario supported by a previous study showing that overactivation of the Wnt pathway can compensate for reduced EGF-Ras pathway activity (Gleason et al., 2002). We therefore tested whether the observed starvation suppression of *lin-3/egf(rf)* depended on Wnt signaling (Supplemental Data, section 2.3). Consistent with this hypothesis, we found that suppression of *lin-3/egf(rf)* mutation was abolished by a null mutation in *bar-1/*β-catenin. However, the suppression effect was also abolished in the double mutant *let-23/egfr(sy1); lin-3/egf(n378)*. Hence, starvation suppression of *lin-3/egf(rf)* does not solely depend on Wnt signaling, but further requires basal Ras activity (Supplemental Data, section 2.3).

Concerning mutations in the Notch pathway, starvation and dauer-inducing environments drastically modified the phenotype of two weak gain-of-function mutations in *lin-12/Notch* (alleles *n302* and *n379*) (Figures 3C; Figures S6C and S6D). Such mild Notch overactivation causes loss of the anchor cell, the source of the LIN-3/EGF inductive signal, without inducing ectopic 2° fates (e.g., Greenwald and Seydoux, 1990), resulting in strong hypoinduction under standard conditions. In contrast, starvation and dauer-inducing environments induced a highly penetrant, hyperinduced phenotype, with P3.p–P8.p frequently adopting 2° fates (as inferred from lineage analysis). Using a transgenic line carrying an anchor cell marker, *cdh-3::gfp* (Inoue et al., 2002), we further confirmed that this environmental effect occurred in the absence of the anchor cell. Therefore, the environment modulated Notch activity in the absence of Ras pathway activation through LIN-3/EGF signaling.

Our assay also included 17 silent mutations that show a wild-type vulval phenotype in the standard environment. Such mutations, most of them in regulators of the Egf/Ras/MAPK pathway, have been shown to alter corresponding pathway activities without disrupting correct vulval pattern establishment (for references, see Table S1). Four of these mutations were sensitive to the environment, including the *unc-101* mutant (this gene encodes a clathrin adaptor protein that negatively regulates LET-23/EGFR; Lee et al., 1994), which showed mild yet consistent hyperinduction in the starvation environment (Figure S5C). Such regulatory elements therefore may appear redundant in standard laboratory conditions, but become functionally essential in maintaining a correct phenotype in other environments.

The results of our mutant assay shows that environmental inputs exert diverse and great modulatory effects when the vulval genetic network is mutationally compromised in any one of the three signaling pathways (Ras, Notch, Wnt).

Activity and Interplay of Vulval Signaling Pathways in Different Environments

In order to examine environmental effects on the wild-type vulval signaling network, we next analyzed how the environment influences the activity and spatiotemporal dynamics of Ras (Yoo et al., 2004) and Notch (Berset et al., 2001) pathways using transcriptional reporter genes (Supplemental Data, section 1.5). We quantified reporter gene activities in the N2 isolate in standard and starvation environments at 20°C during early vulval fate patterning, from mid-L2 to early-L3 stages (after starved worms had been returned to food). Starvation affected the activities of both pathways, causing a stronger activation of the Ras pathway in P6.p and a stronger activation of the Notch pathway in P5.p and P7.p (Figures 4; Figures S9 and S10). In the starvation environment, the spatial expression pattern of Ras versus Notch activities was also generated at an earlier developmental stage. In the starvation environment, we also detected significantly increased expression levels of a major transcriptional target of both Ras and Wnt pathways, *lin-39* (Wagmaister et al., 2006). This starvation effect caused elevated *lin-39* expression in P5.p, P6.p and P7.p throughout the vulval patterning process (mid-L2 to early-L3 stage) (Figure S11). The observed increase in the activity of the Ras pathway produced by starvation could directly explain the reduced effect of hypomorphic mutations (*lin-3/egf* and *let-23/egfr*) in the same pathway (Figures 3B, Figures S3 and S4). Consistent with this hypothesis, we found that starvation induced higher levels of expression of the Ras pathway reporter in a *lin-3/egf(rf)* background (data not shown). Together, these results demonstrate that activity levels of Ras and Notch pathways, as well as the total amount of inductive signal, may vary significantly depending on the environment.

Although environmental variation provoked marked changes in the expression of genes involved in vulval patterning, this only rarely translated into phenotypic variation. Specifically, the starvation environment consistently increased inductive signaling, yet hyperinduction in this environment occurred in only 4 of 1000 wild-type N2 individuals, likely without causing a nonfunctional vulval organ (Figure 2C). As such, the vulval developmental system is able to tolerate considerable quantitative changes in pathway activity caused by environmental variation.

Intra- and Interspecific Variation in Developmental Precision and Errors

Previous work has shown that the canonical vulval cell fate pattern is invariant within the *Caenorhabditis* genus (Félix, 2007). However, it remained unclear as to what extent the precision of the patterning process was evolutionarily conserved. We therefore tested how the genetic background modifies developmental precision and deviations in different environments. We first examined the *C. elegans* isolate JU258 (from Madeira, Portugal) in the six environments (Figure S12). While both JU258 and N2 isolates showed few vulval defects, they differed in type and frequency of deviations in a given environment. In the starvation environment, JU258 displayed a high proportion of individuals with undivided or missing precursor cells (Figures S12D and S12E). In addition, several developmental deviations observed in N2 (hypoinduction, supernumerary 3° cell divisions, defects in vulval morphogenesis) were never found in JU258. The different types of deviant vulval patterns between N2 and JU258 illustrate that the same environmental condition may cause very different developmental challenges depending on the genetic background.

We next followed vulval patterning of three *C. elegans* and three *Caenorhabditis briggsae* wild isolates in the standard and starvation environments. The frequency of deviations from the canonical pattern increased upon starvation and varied among different genotypes (Figures 5A and 5B; Figure S13). For example, the *C. briggsae* isolate HK104 specifically showed an increased frequency of individuals with an undivided P8.p cell in the starvation treatment (Fisher's exact test: $p < 0.0001$, for

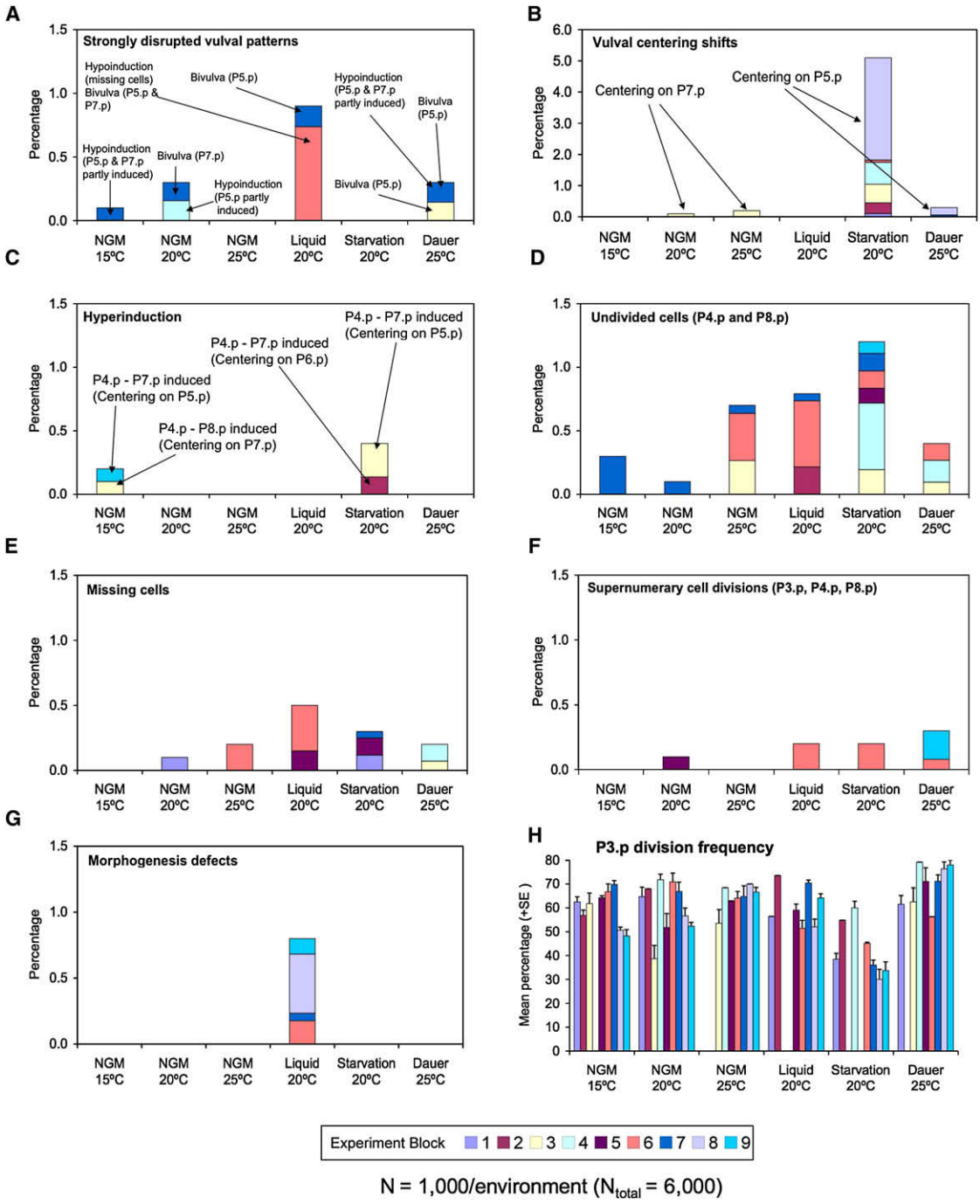

Figure 2. Precision and Deviations of Vulval Cell Fate Patterns in Different Environments Examined in the *C. elegans* Isolate N2

Bars represent the percentage of individuals with the developmental deviation in a given environment (n = 1000 individuals/environment). The relative frequencies of developmental deviations observed in different experimental blocks are distinguished by different colors (for graphical representation, different replicates within a given environment were pooled). The frequency of certain types of developmental deviations (e.g., centering shifts in the starvation environment or P3.p division frequency) showed considerable variation among experimental blocks. Such variation may have been due to differences among populations initiated separately for each experimental block. Different developmental deviations also occurred within the same experimental block in a single environment, suggesting that vulval patterning was sensitive to stochastic variation. Note that we cannot rule out that certain infrequently observed developmental deviations may sometimes have been caused by germ line mutation. However, we found that individuals with a given developmental deviation, such as vulval centering on P5.p, did not consistently generate offspring with the same deviation at high frequency (data not shown).

(A) Strongly disrupted vulval patterns likely leading to a nonfunctional vulva. These patterns included hypoinduction (fewer than three vulval cells) and lineage errors (e.g., defects in lineage orientation causing a Bivulva phenotype [Inoue et al., 2004]).

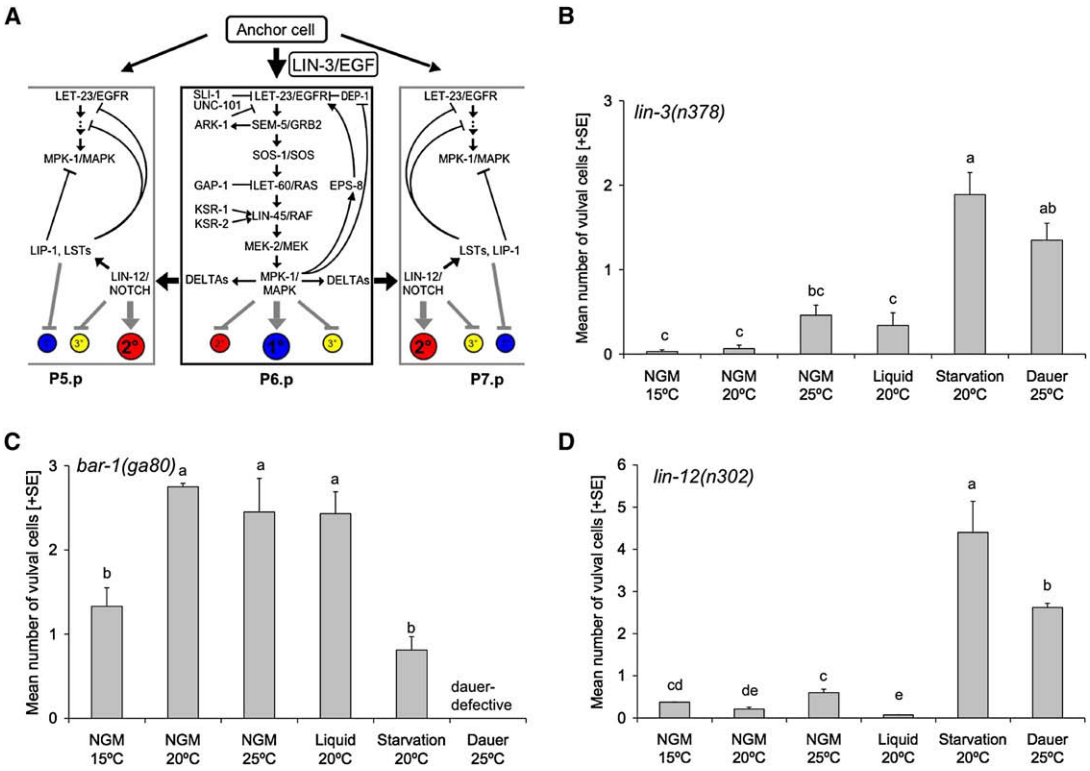

Figure 3. Environmental Sensitivity of the Vulval Signaling Network

(A) Overview of the vulval signaling network with emphasis on feedback loops and crosstalk between components of the EGF/Ras/MAPK and delta/Notch pathways (Sternberg, 2005). The core components of the Ras pathway are shown in the center, and mutations in regulators are indicated laterally. In addition, a Wnt pathway and SynMuv genes are involved in vulval cell fate determination (not shown). The site of action of the Wnt pathway involved in vulval competence and induction is not clearly resolved. Several members of different SynMuv classes (A, B, C) have been shown to act as transcriptional repressors of LIN-3/EGF in hyp7, the epidermal syncytium surrounding the vulval precursor cells, whereas some may act in the Pn.p cells (Fay and Yochem, 2007).

(B–D) Environmental effects on vulval mutant phenotypes. Bars indicate the mean number of induced vulval cells (representing the grand mean of replicate means for a given environment). The mean number of induced vulval cells was not significantly different in environments labeled with the same letter (Tukey's HSD, $p < 0.05$). For details on sample sizes, replicates, and statistical tests, see the Supplemental Data, section 1.2, and Figures S3, S6, and S7. (B) *lin-3(n378)*, a reduction-of-function mutation in the gene encoding the EGF-like anchor cell signal (Ferguson and Horvitz, 1985) (ANOVA, effect environment: $F_{5,272} = 15.98$; $p = 0.0003$). (C) *bar-1(ga80)*, a null mutation in the β-catenin homolog regulating vulval cell competence and induction (Eisenmann et al., 1998) (ANOVA, effect environment: $F_{5,202} = 206.07$; $p < 0.0001$). (D) *lin-12(n302)*, a mild gain-of-function mutation of the Notch homolog causing loss of the anchor cell in all environments (Greenwald and Seydoux, 1990) (ANOVA, effect environment: $F_{4,264} = 29.39$; $p < 0.0001$). Error bars represent 1 SEM.

each of the three experimental blocks). This indicates that the buffering capacity of a given Pn.p cell (i.e., its potential to replace another nonfunctional Pn.p cell) depends on both environment and genotype.

Wild isolates showed pronounced differences with respect to vulval centering shifts: in the starvation environment, centering on P5.p increased in *C. elegans* N2 (Figures 2B and 5B), while centering on P7.p increased in *C. briggsae* JU439 and AF16

(B) Vulval centering shifts. P5.p vulval centering occurred more frequently in the starvation environment (six out of nine experimental blocks) than in the standard NGM environment at 20°C (zero out of nine experimental blocks) (Fisher's exact test, $p = 0.009$). Note that the scale of the y axis in this panel differs from the one in other panels.

(C) Hyperinduction: more than three Pn.p cells were induced, in some cases combined with vulval centering shifts. A total of 6 out of 6000 individuals, restricted to starvation and 15°C environments, exhibited more than three vulval cells. In all cases, a correct 2°-1°-2° vulval pattern was formed, with an additional precursor cell adopting a vulval fate (as shown in Figure 1F).

(D) Undivided cells (P4.p and P8.p.): one or two of the cells remained undivided and fused with the hypodermis.

(E) Missing cells. One or more vulval precursor cells were missing. We could not always infer which exact Pn.p cell was missing, but the presence of more than three competent cells allowed formation of a correct 2°-1°-2° vulval pattern. Missing cells occurred at similar frequencies for anterior Pn.p cells (P3.p–P5.p) and posterior cells (P7.p and P8.p).

(F) Supernumerary cell divisions (P3.p, P4.p and P8.p.): vulval precursor cells with a nonvulval fate and more than two cell progeny.

(G) Defects in vulval morphogenesis. L4-stage individuals showed wild-type vulval induction (3.0 induced cells), but the progeny of P5.p or P7.p was not correctly adhering to the cuticle, thus likely leading to defects in morphogenesis. However, we could not establish whether this developmental deviation led to a nonfunctional vulva.

(H) P3.p division frequency. The propensity of P3.p to adopt 3° versus F fates varied significantly among environments (GLM, main effect *environment*: df = 5, $\chi^2 = 56.11$, $p < 0.0001$) and also among experimental blocks, as indicated by the significant interaction term (GLM, interaction effect *environment* × *block*: df = 40, $\chi^2 = 83.82$, $p < 0.0001$; main effect *block*, df = 8, $\chi^2 = 10.66$, $p = 0.22$). The starvation environment caused a consistently decreased P3.p division frequency.

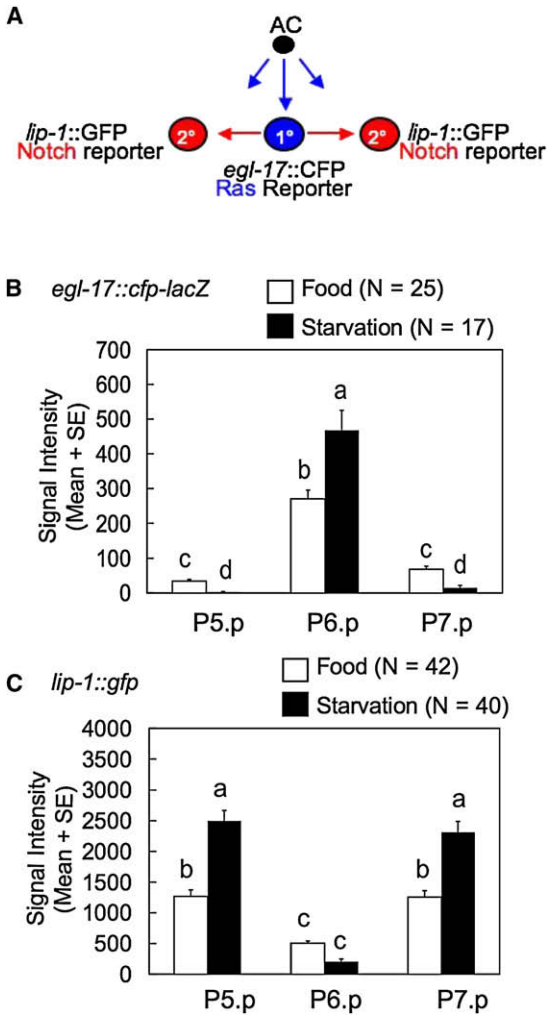

Figure 4. Activity and Interplay of Vulval Signaling Pathways in Different Environments

Quantification of transcriptional reporter activities at the lethargus L2/L3 stage in food and starvation environments (for other developmental stages, see Figure S9 and S10). Bars labeled with the same letter did not show significantly different expression levels (Tukey's HSD, $p < 0.05$). For complete ANOVA model and results, see Table S2). Note: the differential response of Ras versus Notch reporter in the same Pn.p cells showed that the starvation environment did not cause a nonspecific, overall increase in fluorescent reporter intensity.

(A) Schematic drawing of LIN-3/EGF (blue arrows) and delta (red arrows) intercellular signaling, and of their downstream transcriptional targets assayed via fluorescent reporters in the receiving cells.

(B) Mean signal (pixel) intensity of the Ras pathway reporter, *egl-17::cfp-LacZ* (Yoo et al., 2004), in P5.p–P7.p.

(C) Mean signal (pixel) intensity of the Notch pathway reporter, *lip-1::gfp* (Berset et al., 2001), in P5.p–P7.p. Error bars represent 1 SEM.

(Figure 5B; data not shown). Such shifts likely arose during the alignment process between anchor cell (the LIN-3/EGF-releasing cell, born in the early-L2 stage) and vulval precursor cells, during which the anchor cell induces the closest Pn.p cell to adopt the central 1° vulval fate (usually P6.p). By measuring relative distances between anchor cell and P5.p versus P7.p in the L2 stage (Figure 5C; Supplemental Data, section 1.6), we found that it was generally closer to P5.p in *C. elegans* N2, while it was closer to P7.p in *C. briggsae* AF16 (Figure 5C). Since LIN-3/EGF from the anchor cell plays a similar inductive role in both species (Félix, 2007), N2 individuals would be expected to be more likely to show centering shifts toward the anterior, and AF16 individuals toward the posterior (however, we cannot exclude that centering shifts may have been caused by additional factors, such as variation in LIN-3/EGF sensitivity among different Pn.p cells). The direction of these vulval centering shifts may interplay with observed differences in competence group properties between these two species (Delattre and Félix, 2001; Félix, 2007): in N2, upon ablation of the anchor cell and ubiquitous ectopic expression of LIN-3/EGF, the anterior cell P4.p adopts a vulval fate more frequently than the posterior cell P8.p (Clandinin et al., 1997; Katz et al., 1995). Conversely, in the same experiment in AF16, P4.p adopts a vulval fate less frequently than P8.p (Félix, 2007). In addition, P3.p is competent in *C. elegans*, but not in *C. briggsae* (Delattre and Félix, 2001). The observed evolutionary variation in the propensity for anterior versus posterior centering shifts thus coincides with evolutionary variation in the competence level of anterior versus posterior Pn.p cells.

To test how the environment affected the fate patterning process of different wild genotypes, we introgressed the *lin-3/egf* reduction-of-function mutation (allele *n378*), causing a highly penetrant, hypoinduced vulval phenotype in the standard environment, into six genetically divergent *C. elegans* wild isolates (Supplemental Data, section 1.9). We assayed the phenotypic effect of this mutation in four environments and found that the genetic background had a significant effect on the penetrance of this mutation. This effect was further modified by the environment, as revealed by strong genotype-by-environment interactions (ANOVA, genotype × environment interaction, $F_{15,1415} = 2.90$; $p = 0.0002$) (Figure S14). The starvation suppression of *lin-3/egf* occurred in all isolates, but dauer suppression was limited to N2. Environmental effects on the penetrance of a given vulval mutation therefore show genetic variation, suggesting evolution of the interactions between vulval signaling network and the environment. Consequently, robustness to specific environmental and mutational challenges may evolve.

These comparative analyses show that vulval development leads to a precise and nearly invariant pattern output in all tested *Caenorhabditis* wild isolates, yet the frequency and type of "errors" in the patterning output are subject to evolutionary change. These results indicate that the developmental processes affecting vulval formation show cryptic evolution within and between different *Caenorhabditis* species.

Degradation of Developmental Precision in Mutation Accumulation Lines

Robustness is often regarded as an adaptive system feature without also considering possible nonadaptive processes underlying developmental system evolution (Lynch, 2007). To address this problem, we next asked to what extent the observed robustness of *C. elegans* vulval development might be adaptive. Importantly, the vulva is a fitness-related phenotype, and we therefore expect the vulval cell fate pattern (2°-1°-2°) to be a trait under stabilizing selection. This expectation agrees with three observations. First, deviations from the canonical pattern occur rarely, even under stressful conditions. Second, the vulval phenotype is evolutionarily conserved: P(5–7).p form the vulva of

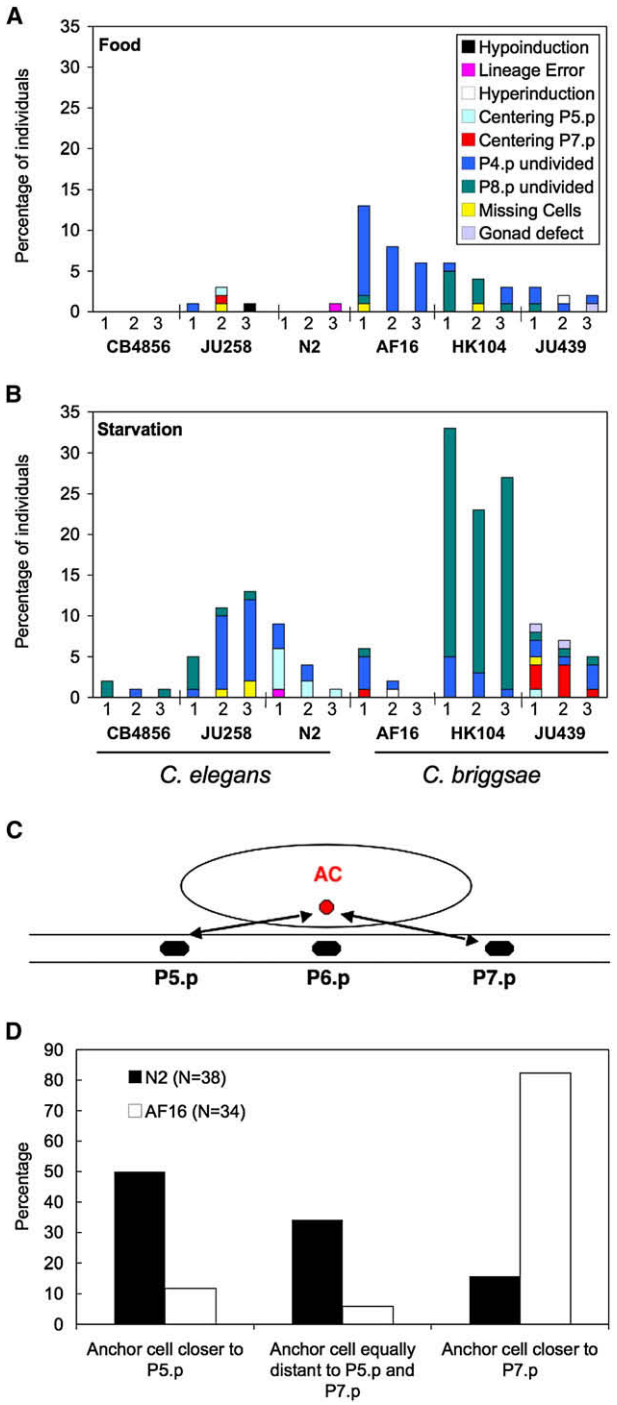

Figure 5. Precision and Deviations in Vulval Cell Fate Patterns of *C. elegans* and *C. briggsae* Isolates in Standard Versus Starvation Environment

(A and B) For each of three experimental blocks, six isolates of *C. elegans* and *C. briggsae* were assayed in food and starvation environments in parallel (n = 100/isolate/environment/block; n_{total} = 3600). Bars represent the percentage of individuals with developmental deviations, which are distinguished by different colors. For each isolate, the results of experimental blocks 1–3 are listed sequentially. (A) Food environment (standard NGM plates). Note that *C. briggsae* isolates showed an increased frequency of undivided P4.p or P8.p in this standard condition as compared with *C. elegans* isolates. (B) Starvation environment (48 hr of starvation during the early-L2 stage). Several developmental deviations were more frequently found after starvation; however, this response was genotype specific. For data on P3.p division frequency, see Figure S13.

(C and D) Anchor cell-vulval precursor cell alignment process. (C) Schematic drawing showing the gonadal anchor cell and the vulval precursor cells in the mid-L2 stage (isolate N2). Arrows depict the distance measurements taken between the anchor cell (AC) and P5.p and P7.p (see Supplemental Data, section 1.8). (D) Differences in anchor cell position during the L2 stage in individuals of *C. elegans* N2 versus *C. briggsae* AF16. In 19 of 38 N2 individuals, the anchor was closer to P5.p than to P7.p (with 13 individuals having an anchor cell equidistant to P5.p and P7.p), whereas only 4 of 34 of the AF16 individuals had anchor cells closer to P5.p (with 28 of 34 individuals having an anchor cell closer to P7.p than to P5.p; 2 of 34 individuals had an anchor cell equidistant to P5.p and P7.p). The proportions of individuals showing an anchor cell position bias toward P5.p were significantly different between N2 and AF16 (Fisher's exact test, p = 0.0008).

all *Caenorhabditis* species in the same spatial pattern and P3.p, P4.p, and P8.p adopt nonvulval fates (Félix, 2007). Such extensive evolutionary stasis is typical of a trait under strong stabilizing selection (Hansen and Houle, 2004). Third, we found that the precision of vulval patterning degraded when selection was relaxed due to the accumulation of spontaneous mutations (Figure 6; Table S4). Regarding this last point, we examined lines derived from N2 that had accumulated mutations for 400–430 generations (Vassilieva et al., 2000). DNA sequencing revealed that these lines accumulated approximately one mutation per haploid genome per generation (Denver et al., 2004). The proportion of lines showing one or more individual with developmental deviations (including nonfunctional patterns) was significantly higher in mutation accumulation (n = 28/54) than in control N2 lines (n = 4/20) (Fisher's exact test, p = 0.0253) (see also Figure 6). Frequent deviations included the loss of competence of one or more Pn.p cells (missing cell or early fusion), vulval centering shifts, as well as hypoinduction. These developmental deviants were therefore readily induced by random mutation in the N2 isolate. When examining natural wild isolates (N2 and other isolates) in the same standard environment (Delattre and Félix, 2001), such developmental deviations occurred very rarely despite the presence of substantial genetic variation among isolates (Dolgin et al., 2007). Mutations triggering such developmental deviations (even at very low frequencies) are therefore apparently counterselected in the wild. These results also provide evidence that selection maintains competence of additional Pn.p cells (P4.p and P8.p). Hence, precision and robustness of vulval development are likely under selection.

Conclusions

As for many other developmental processes (Wagner, 2005), *C. elegans* vulval cell fate patterning has been inferred to be precise and robust, primarily based on mutant analyses and modeling approaches (Braendle et al., 2008; Giurumescu et al., 2006; Kenyon, 1995; Sternberg, 2005). We experimentally confirm that a functional vulval pattern is robustly generated in the wild-type condition across a range of environments, but we further show that this robustness has limits: while certain deviant patterns (e.g., a missing cell or vulval centering shifts) can be rescued to generate a functional pattern (2°-1°-2°), environmental (and stochastic) factors may induce combinations of such

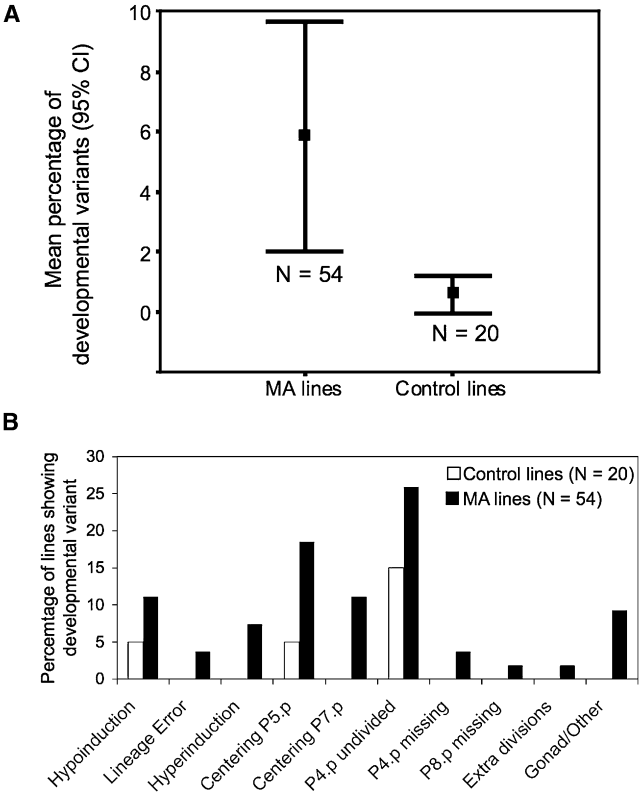

Figure 6. Degradation of Developmental Precision in Mutation Accumulation Lines

We determined the proportion of vulval developmental variants deviating from the canonical wild-type pattern in mutation accumulation (MA) lines (Vassilieva et al., 2000) (n = 54) versus control N2 lines (n = 20) in standard laboratory conditions at 20°C. For each MA line, 41–66 individuals (n_{total} = 2658) were scored; and for each control line, 50 individuals (n_{total} = 1000) were scored. Detailed results for individual lines are represented in Table S4.

(A) The mean proportion of developmental deviations was higher in MA lines (range, 0.0%–71.4%) compared with control lines (range, 0.0%–4.0%) (Mann-Whitney U test, U = 340.0; p = 0.007; n = 74).

(B) Types of observed vulval developmental deviations and proportion of lines showing one or more individuals with such a deviant cell fate pattern. Bars represent 95% confidence interval.

deviations, leading to nonfunctional cell fate patterns, such as hypoinduction. The occurrence of a number of deviant yet functional vulval patterns shows that cellular redundancy among Pn.p cells competent to adopt a vulval fate is one apparent mechanism contributing to the environmental robustness of this developmental system. This cellular redundancy is controlled by the expression domain of the Hox gene *lin-39* (Eisenmann et al., 1998).

Although the vulval phenotype remained nearly invariant across tested environments, we found that the underlying molecular processes were environmentally sensitive. Conditions affecting the nutritional status of the animal (i.e., starvation and dauer-inducing environments) had the most drastic, and sometimes distinct, effects in both *C. elegans* and *C. briggsae*. That fluctuation in food availability is indeed an important ecological variable is indicated by the natural *Caenorhabditis* habitat, which is highly ephemeral in food sources (Barrière and Félix, 2005). Moreover, dauer larvae are the predominant developmental stage found in natural populations, and isolated non-dauer individuals often show signs of malnourishment (Barrière and Félix, 2005). Our study and previous results (Battu et al., 2003; Ferguson and Horvitz, 1985; Moghal et al., 2003) suggest that such ecologically relevant stimuli modulate vulval signaling pathways. Here we specifically show that activity levels of Ras and Notch pathways involved in vulval cell fate patterning were significantly higher in the starvation environment; however, such quantitative variation was tolerated by the system and rarely caused corresponding defects in the cell fate pattern output. The observed phenotypic insensitivity to changes in molecular pathway activities likely result from two prominent features of the vulval signaling network: buffering of quantitative variation through switch-like behaviors (e.g., due to high cooperativity and positive feedback loops) (Nijhout and Berg, 2003; Stetak et al., 2006; Yoo and Greenwald, 2005) and crosstalk among Ras and Notch pathways that results in stable discrimination of 1° and 2° vulval fates (Berset et al., 2001; Giurumescu et al., 2006; Yoo et al., 2004). Tolerance to pathway fluctuations caused by the environment may further be enhanced by specific regulatory elements, as suggested by the example of UNC-101, a negative regulator of the Ras pathway. In addition, compensatory interplay between Ras and Wnt pathways, which show partial redundancy in inducing vulval fates (Gleason et al., 2002), may ensure sufficient levels of inductive signaling when one of the pathways is compromised either by mutation (Gleason et al., 2002) or the environment (Moghal et al., 2003; the present study). Robustness of the vulval developmental system to environmental variation therefore results through an integration of *multiple* buffering capacities at the molecular and cellular level.

All examined *Caenorhabditis* isolates and species showed few errors in vulval formation, but they differed in the spectrum of rare developmental deviants and errors generated in response to the environment. This type of variation can be explained by genetic divergence in certain developmental mechanisms (e.g., the alignment process between anchor cell and vulval precursor cells), which, under normal laboratory conditions, do not result in variation of the final vulval cell fate pattern. Such cryptic evolution of the vulval developmental system seems to be prevalent and has been revealed among different *Caenorhabditis* species (Félix, 2007) and among different *C. elegans* isolates (J. Milloz and M.-A.F., unpublished data), as well as in *Pristionchus* nematodes (e.g., Zauner and Sommer, 2007). Although we cannot infer the causes for the extensive cryptic evolution of nematode vulval development, the findings are consistent with the hypothesis that the environmental robustness of this process may confer robustness to mutations. In turn, such mutational robustness would result in genetic divergence of underlying developmental mechanisms without generating changes in the final phenotype (Gibson and Dworkin, 2004; Wagner, 2005). Importantly, our results indicate the limits of such "developmental system drift" (True and Haag, 2001), as most developmental variants deviating from the canonical vulval pattern are counterselected. Thus, interplay of such phenotypically neutral drift and selection likely generates evolutionary divergence in the developmental system, so that different genotypes may rely on different mechanistic solutions to provide the same system output.

Because the environment modulates diverse parameters during vulval development, such as cell-cell interactions or interplay of involved signaling pathways, environmental variation may further accelerate evolution of this developmental system. Such evolution may be partly adaptive, as different environments impose different challenges to the vulval developmental system. As a consequence, different environmental conditions may result in selection for alternative mechanistic properties conferring developmental robustness. Gaining insights into the poorly understood ecology of *C. elegans* and related nematodes may therefore prove necessary to elucidate the full evolutionary and functional significance of mechanisms underlying vulval development.

EXPERIMENTAL PROCEDURES

Strains and Experimental Procedures

Previously described methods were used to maintain cultures and to perform genetics analyses of *C. elegans* and *C. briggsae* strains (Wood, 1988). All experiments testing for differences in environmental effects (wild-type, mutant and reporter assays) were performed in parallel, and generally repeated several times. Prior to each experiment, a single individual was isolated (from freshly thawed worm stock kept at −80°C) to initiate the experimental population, which was kept under standard conditions for four to six generations prior to the experiment. Individuals used for a given experiment were derived from identical maternal and grandmaternal conditions and randomly allocated to the different test environments. For details, see Supplemental Data, section 1.

Scoring of Vulval Phenotype

The vulval cell fate pattern was determined at the L4 stage after Pn.p divisions using Nomarski microscopy on individuals anaesthetized with sodium azide (Wood, 1988). We examined overall morphology of the vulval pattern and counted the number of Pn.p cell progeny. The fates of the cells P3.p–P8.p were determined as described previously (Sternberg and Horvitz, 1986). For characteristics of the different cell lineages, see legend for Figure 1. For definitions of variant vulval pattern and abnormal vulval lineages, see legend of Figure 2.

Statistical Analyses

Statistical tests were performed using the software programs JMP 6.0 or SPSS 11.0 for Macintosh. For parametric tests, data were transformed (usually box-Cox transformations) where necessary to meet the assumptions of ANOVA procedures (homogeneity of variances and normal distributions of residuals). For post hoc comparisons, Tukey's honestly significant difference (HSD) procedure was used. For data, where ANOVA assumptions could not be met, we used nonparametric tests (e.g., Mann-Whitney U test). For comparison of proportional data, we used semiparametric tests (GLMs) and nonparametric tests, such as Fisher's exact test where appropriate (all two-tailed). All errors of the mean, including error bars in figures, represent the standard error of the mean. For details on statistical analyses of different experiments, see Supplemental Data, section 1.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental Results, fourteen figures, four tables, and Supplemental References and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/15/5/714/DC1/.

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