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Robustness and evolution: concepts, insights, and challenges from a developmental model system

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Abstract

Robustness, the persistence of an organismal trait under perturbations, is a ubiquituous property of complex living systems. We here discuss key concepts related to robustness with examples from vulva development in the nematode *Caenorhabditis elegans*. We emphasize the need to be clear about the perturbations a trait is (or is not) robust to. We discuss two prominent mechanistic causes of robustness, namely redundancy and distributed robustness. We also discuss possible evolutionary causes of robustness, one of which does not involve natural selection. To better understand robustness is of paramount importance for understanding organismal evolution. Part of the reason is that highly robust systems can accumulate cryptic variation that can serve as a source of new adaptations and evolutionary innovations. We point to some key challenges in improving our understanding of robustness.

Introduction

Here we first define robustness and review experimental ways to detect it. We then discuss the proximate mechanisms underlying robustness. We finally discuss evolutionary causes and consequences of robustness.

What is robustness, and why is it important?

Robustness is the persistence of an organismal trait under perturbations. Many different organismal features could qualify as *traits* in this definition of robustness. A trait could be the proper fold or activity of a protein, a gene expression pattern produced by a regulatory gene network, the regular progression of a cell division cycle, the communication of a molecular signal from cell surface to nucleus, a cell interaction necessary for embryogenesis, or the proper formation of a viable organism or organ.

Robustness is important in ensuring the stability of phenotypic traits which are constantly exposed to genetic and non-genetic variation. To better understand robustness is of paramount importance for understanding organismal evolution, because robustness permits cryptic genetic variation to accumulate. Such variation may serve as a source of new adaptations and evolutionary innovations.

We will here focus on developmental traits and on the robust formation of organs. Specifically, we will discuss important concepts and challenges in studying robustness using the vulva of the nematode *C. elegans,* an exceptionally well-studied developmental model. Here, the robust trait is the spatial pattern of vulval cell fates (Box 1). For further reading on different aspects of biological robustness and canalization, a non-exhaustive list of more extensive reviews includes: Gibson and Wagner 2000; Debat and David 2001; de Visser et al. 2003; Gibson and Dworkin 2004; Flatt 2005; Wagner 2005a; Dworkin 2005a.

We note that the final product of a biological process may be robust despite variation in some *intermediate trait* (Fig. 1), such as a developmental stage, the activity of a signaling pathway, or the expression of a gene product. To give but one example from vulval development, animals that are heterozygotes for a null mutation in the gene coding for the EGF signal have a normal vulva fate pattern (Ferguson and Horvitz 1985). This indicates that variation in EGF signal levels – which can be viewed as an intermediate phenotypic trait – is buffered. Cell fate output is invariant to such buffered variation.

Robustness to what?

Robustness can be discussed sensibly only if two cardinal questions have been resolved. What is the trait of interest? And what is the perturbation of interest? There are three principal kinds of perturbations to which a system may be robust: *stochastic noise*, *environmental change*, and *genetic variation* (Fig. 1).

Noise refers to the stochastic fluctuations that occur in any biological system, for example in the concentration of a biological molecule or in a cell's position, either over time, or between two genetically identical individuals, even if the external environment is constant. Developmental traits lacking robustness to noise include human fingerprints, which differ among genetically identical twins (Stigler 1995). An example from *C. elegans* vulval development is the division pattern of the cell P3.p at the anterior border of the vulva competence group (see Box). This cell divides in only some genetically identical worms, whereas in others it directly fuses with the epidermal syncytium and loses vulval competence (Sulston and Horvitz 1977; Eisenmann et al. 1998).

The second kind of perturbation is variation in the external environment, for example a change in temperature, salinity or nutrient availability. Many developmental traits, such as the *C. elegans* vulva fate pattern (C. Braendle and M.-A. F., unpublished), are highly robust to environmental changes. In contrast, some traits are strongly influenced by the environment, for example the propensity of *C. elegans* larvae to develop through the resistant dauer stage (Riddle and Albert 1997). The effect of the environment may range from a shift in a quantitative distribution (for example body size as a function of nutrition in humans) to the appearance of alternative phenotypes (for example caste determination as a function of nutrition in social insects). In these cases, the final phenotype is not robust, but plastic (Pigliucci 2005). The ecology of an organism is thus clearly important to understanding a trait's robustness properties. Specifically, robustness to frequent environmental perturbations may be of greater adaptive significance than robustness to perturbations that occur rarely or never.

The third kind of perturbation is genetic change, either through *de novo* mutation or through recombination. Here, the genetic structure of populations becomes relevant to characterize robustness properties. As a simple example, in diploids the effect of a new recessive mutation will depend on its probability to be found in the homozygous state. This probability itself is a function of the mode of reproduction (selfing versus outcrossing) and of the effective size of the population (Hartl and Clark 1997). In addition to mutational variation, robustness to genetic variation includes robustness to the effect of recombination between alleles at different loci. As a consequence, spatial genetic structure becomes crucial in the evolution of a system's robustness properties, for example through the migration rate between populations adapted to local environments (Ancel Meyers and Bull 2002; Proulx and Phillips 2005). Frequent recombination may favor the evolution of mutational robustness. This form of genetic robustness may result in negative epistasis (synthetic effects of deleterious mutations), which in turn renders sex (and recombination) advantageous. This feedback between genetic robustness and recombination frequency has been proposed as an explanation for the evolution and maintenance of sex (Azevedo et al. 2006).

How is robustness detected?

Robustness is not an all-or-nothing property. It is a matter of *degree.* For a quantitative trait, lack of robustness can be expressed using the coefficient of variation (square root of the variance over the mean) for the trait or, when comparing two conditions, the unsigned difference in the means (Dworkin 2005a). For a complex qualitative trait such as a protein sequence or the vulval cell fate pattern, robustness (or a lack thereof) can be expressed using the proportion of deviant phenotypes produced in response to perturbations. For example, a given environmental condition or mutation may produce a deviant phenotype for a large (e.g., 10^{-2}) or small (10^{-10}) fraction of organisms. In addition, the *types of deviation* ("errors") that a system produces – an amino-acid misincorporation in a protein sequence during translation, a deviant cell fate pattern (see Box) or the shape of an organ – and their consequence on the organism's fitness influence crucially how natural selection acts on a system, yet they are often not investigated. We now outline three basic experimental approaches to probe and measure robustness, following the distinction between the three different kinds of perturbations that may affect a system.

Robustness of a trait to noise is best detected by assaying individuals of an isogenic strain in a given constant environment. The use of isogenic strains eliminates confounding effects from genetic variation between individuals in assessing the effect of stochastic noise. For organisms that have a prominent haploid life cycle stage (many fungi, bacteria) or are commonly selfing (such as *C. elegans*), isogenic strains are easy to obtain. Vulva development of *C. elegans* has been mostly studied using the isogenic N2 reference strain in one standard culture condition. In these conditions, vulva cell fate patterning errors are found at a low frequency (on the order of 10^{-3} or less, for deviations that disrupt the cell fate pattern, but do not necessarily prevent egg-laying), implying that this aspect of vulva development is precise and robust to stochastic noise (Delattre and Félix 2001; C. Braendle and M.-A. F., unpublished). The degree of robustness and the types of error can be compared between different isogenic backgrounds. A second way to eliminate confounding effects from genetic variation in measuring robustness to noise is to quantify the developmental variation between the right and left sides of an animal (fluctuating asymmetry).

Robustness of a trait to environmental variation is detected by subjecting organisms to a given environmental change or an array of environmental changes that may mimic ecologically relevant environments, possibly including some "stressful" environments. In the vulva example, under starvation conditions in the second larval stage (one test environment), *C. elegans* N2 individuals are prone to miscenter their vulva on P5.p instead of P6.p (C. Braendle and M.-A. F., unpublished). This centering variation of the cell fate pattern results in a quasi-normal vulva because P4.p is competent to form vulval tissue and adopts a 2° fate in these animals. Furthermore, the incidence and patterning of vulva variants vary with environmental conditions. They also vary with the wild-type genetic background (C. Braendle and M.-A. F., unpublished), which means that they are subject to evolutionary change, possibly via the action of natural selection (see below).

Robustness to a given mutation is detected by comparing the mutant to the reference wild type genotype, and asking whether the mutation is silent or neutral, that is, whether it lacks an effect on the trait. The question whether a mutation is truly neutral is surprisingly difficult to answer (Wagner 2005b). For instance, a mutation might have an effect at one developmental stage, but not on the final phenotype (Fig. 1C), or vice versa. In addition, a mutation's effect may critically depend on the genotype at other loci. For instance, in *C. elegans* vulva development, null mutations in the gene coding for the Ras GTPase activating protein (GAP-1, a Ras inhibitor), or for an activator of EGF receptor degradation (SLI-1), are silent with respect to the final cell fate pattern. The system is

robust to these mutations. In contrast, the double mutant displays an excess of vulval fates, showing that these two molecules indeed modulate Ras pathway activity and are thus not silent at this level (Yoon et al. 1995; Hajnal et al. 1997; Hopper et al. 2000).

This test of robustness to a given mutation can be extended to a statistical measure (e.g. the mutational variance for quantitative traits; Lynch et al. 1999) of the effect of thousands of random mutations that are produced either spontaneously or through systematic mutagenesis studies. Systematic gene inactivation libraries (for example RNAi libraries in *C. elegans* (Kamath et al. 2003) are becoming available in several organisms. However, many of these "inactivations" may be partial and result in a reduction of a gene's function. They thus only represent a narrow band within a broader spectrum of mutational effects in the wild. More "natural" mutational patterns are best reconstituted using spontaneous mutation accumulation lines (Denver et al. 2004). These lines are obtained by propagating multiple populations (lines) of organisms by only retaining one or two randomly chosen individuals per line for reproduction at each generation. The resulting severe bottleneck reduces the efficacy of natural selection and allows the accumulation of deleterious mutations over many generations (Lynch et al. 1999). The phenotypic effect of random mutation on the vulva system was probed using a set of mutation accumulation lines derived from the N2 genotype over the course of 400 generations (a generous gift from L. Vassilieva and M. Lynch; Vassilieva et al. 2000): "errors" in cell fate patterning and centering increased in most of the lines compared to the N2 control (M.-A. F., unpublished).

Another, indirect approach to inferring robustness to genetic change uses genetic variation that occurs in natural populations. In this comparative approach, one considers genetic variation among individuals of the same or different species. These species share an invariant trait that may be produced by a varying developmental process. For example, in several species related to *C. elegans* the final vulval cell fate pattern is invariant, but the developmental route to this final pattern varies strikingly among them (Box 1, Fig. B1) (Félix 1999; Sommer 2000). This qualitative approach is powerful because it allows the comparison of organisms and genotypes that are only remotely related. Such organisms have accumulated much greater genetic change than can be produced in laboratory evolution experiments. However, the approach does not provide a quantitative measure of robustness to random genetic change. It also has the disadvantage that the adaptive significance of the existing variation (truly neutral, beneficial, or slightly deleterious) is often not known.

Finally, a generic approach to estimating robustness applies to traits whose mechanistic basis is experimentally well-studied. For such traits, one can build quantitative models of the developmental process producing a trait. Such models permit estimation of the trait's sensitivity to changes in model parameters (Barkai and Leibler 1997; von Dassow et al. 2000; Meir et al. 2002; Eldar et al. 2002; Eldar et al. 2003). Changes in parameters (for example, the affinity of a transcription factor for its target site, or the degradation rate of a protein) may result either from environmental variation or from mutational change. To systematically perturb model parameters thus allows one to assay a system's robustness to multiple types of change. One challenge for this approach is to provide a quantitative framework to integrate information about mutational variation and population structure on one hand, and environmental variation on the other. In addition, experimental data for model building and validation are sorely needed.

Proximate (mechanistic) causes of robustness

Different categorizations of mechanistic causes of robustness are conceivable (Gerhart and Kirschner 1997; McAdams and Arkin 1999; Wagner 2005c). We here emphasize a simple yet very fundamental

one: *redundancy* versus *distributed robustness* (Fig. 2).

In a system with redundant parts, multiple components of a system have the same function. Redundancy is generally an important cause of robustness in systems whose parts are genes. The reason is that genomes are littered with duplicate genes, and gene duplication is a process that produces genes with redundant functions. Redundancy may also be found at other levels, for example between cells. An example is the redundancy between cells of the vulval competence group, where one cell can replace another (defective) one in making vulval tissue (Sulston and White 1980).

Distributed robustness, in contrast, can exist even in systems where no two parts exert the same function. Prominent candidate examples of distributed robustness can be found in metabolic systems. For example, many metabolic functions have long feedback loops, where the end-product of a long chain of chemical reaction allosterically inhibits the enzyme catalyzing the first reaction, thus providing homeostatic regulation. Similarly, in complex metabolic reaction networks, blockage of one metabolic pathway may have little consequence if an important metabolite can be produced through an alternative pathway, even though the two pathways may not share a single enzyme with identical (redundant) functions.

Which of these causes of robustness, redundancy versus distributed robustness, is prevalent in biological systems is a matter of some debate. However, the often rapid divergence in both sequence and function of gene duplicates suggests that gene redundancy may be less important in providing robustness than one might think (Wagner 2005c). Although a systematic study of the robustness of altered vulva signaling networks is still missing, the available evidence indicates that distributed robustness is important in vulva development. Specifically, the vulva system appears to have several mechanistic features that involve distributed robustness.

First, the dynamic behavior of core components of the Ras pathway results in non-linearities and may thus contribute to robustness to a broad range of variation in EGF signaling. For example, the multiple phosphorylations of MAP kinase and the positive feedback loop from the activated MAP kinase to the EGF receptor (Box 1, Fig. B2) are likely to create a switch between at least two activity plateaus, a high Ras pathway activity triggering a 1° fate, a low Ras activity a 3° fate.

Second, the Ras pathway has many additional inputs of silent positive and negative regulators that can buffer genetic (or non-genetic) variation (Fig. B2) (Sundaram 2006). As mentioned above with the SLI-1/GAP-1 example, the knockout of one regulator is silent, but the inactivation of two of these regulators may have an effect (Ferguson and Horvitz 1989; Hopper et al. 2000; Kao et al. 2004; Berset et al. 2005). The affected regulators are not redundant, in the sense that they usually do not perform the same molecular activity, nor do they act at the same step in the pathway. One exception is the gene duplication of the positive regulator KSR (Ohmachi et al. 2002).

Third, the cross-talk between the Ras and Notch pathways is a typical case of distributed robustness contributing to the specification of three cell fates (Giurumescu et al. 2006). A high Ras activity triggers Notch degradation in the 1° cell and thus ensures that the cell does not adopt a 2° fate (Shaye and Greenwald 2002). A high Ras activity also activates the expression of several Delta-like molecules (the Notch ligands) by the 1° cell (Chen and Greenwald 2004). The Delta-like molecules activate Notch in neighboring cells, which in turn inhibits Ras pathway activity in those cells (Berset et al. 2001; Yoo et al. 2004). This interaction probably helps form a robust switch between the 2° and 1° fates.

Fourth, at least in some experimental conditions, the 2° vulval fate can be specified either through morphogen action of the EGF inducer at intermediate doses (Katz et al. 1995), or through lateral activation of the Notch pathway by the 1° cell, which itself acts downstream of EGF/Ras signaling in the 1° cell (Koga and Ohshima 1995; Simske and Kim 1995). If developmental perturbations inhibit one mechanism, the alternative

mechanism may guarantee a stable output (Kenyon 1995). Again, these two mechanisms may be said to act redundantly in a wide sense, but they do not perform equivalent activities in the vulva signaling network: one is directly downstream of the EGF inducer, while the other is downstream of lateral signaling through Notch. Overall network topology thus contributes to the robustness of the vulva system.

Clearly, to study the mechanistic causes of robustness is crucial to understand its functional and evolutionary significance. Yet, despite having learnt many mechanistic details about the vulva signaling network or similar models, we still know very little about how the system actually operates in different environmental conditions, what type of noise it is subject to, and when a given regulatory interaction occurs and is required for the final output. This lack of insight challenges us to better characterize the mechanistic causes of robustness in this and other model systems.

Ultimate (evolutionary) causes of robustness

The robustness of a trait to perturbations can have two evolutionary causes. One such cause – you might call it "*robustness for free*" – is rooted in the observation that most biological processes (from enzymatic catalysis to organismal development) have an astronomical number of alternative yet equivalent solutions. These solutions can be thought of existing in a neutral space, in which individual solutions can often be connected through a series of neutral genetic changes (Gavrilets 2004; Wagner 2005a). We use the term 'neutral' in the sense that the change has no effect on the final phenotype because it is very difficult to assess whether any change is neutral for 'fitness' (Wagner 2005a). In other words, the robustness of a trait may simply derive from the existence of many alternative ways of building it. A second possibility is that robustness is an *evolutionary adaptation to perturbations*. Where robustness of a trait is advantageous, natural selection can favor genotypes that render the trait robust. For developmental traits, such evolved robustness is called canalization (Waddington 1942; Gibson and Wagner 2000).

A sizable theoretical literature has arisen around the question under what conditions natural selection will lead to a trait's increased robustness (Wagner 1996; Wagner et al. 1997a; Wagner et al. 1997b; Houle 1998; Krakauer and Nowak 1999; van Nimwegen et al. 1999; Wagner 2000; Wilke 2001; Krakauer and Plotkin 2002; Meiklejohn and Hartl 2002; Siegal and Bergman 2002; Bagheri-Chaichian et al. 2003; Proulx and Phillips 2005). A general insight that has emerged from this theoretical literature is that high robustness can only readily evolve to perturbations that are abundant. Except under high mutation rates, noise and environmental change are likely to be more important driving forces for the evolution of robustness. However, it is likely that the effect of mutation and of non-genetic change on a system are partially correlated, because both affect the same underlying biological processes. For example, an environmental change that results in a higher degradation rate of a protein may have effects similar to that of a reduction-of-function mutation causing a reduced gene expression level or reduced protein activity. In this case, robustness to the environmental change may result in robustness to the genetic change. Obviously, exceptions to this correlation are possible: a given environmental variation and a given mutation may have different effects on a system (Milton et al. 2003; Dworkin 2005b). Unfortunately, a systematic experimental test of the relationship between environmental and genetic robustness of a trait is still lacking.

Despite an abundance of theoretical work, it is currently not clear which of the two potential causes – robustness for free or natural selection – is prevalent. For example, in the vulva system, robustness to stochastic and environmental variations may be an adaptation, the simple result of a selective process eliminating genetic variants that are less robust and thus deleterious in ecologically relevant environments. The comparison of vulva phenotypes in mutation

accumulation lines with those of natural wild strains indeed suggests that several vulva phenotypes are under selection pressure (directly or indirectly), since they are easy to change through mutations yet very rare in the natural wild strains (M.-A.F., unpublished). Some robust features of the vulva network are thus likely to have evolved under selection, rather than merely as an accidental byproduct of the system's architecture. On the other hand, non-linear effects that contribute to robustness may be unavoidable consequences of system properties that were not subject to direct selection on robustness. For example, enzymatic reactions are often relatively insensitive to enzyme concentrations. (Developmental signal transduction pathways involve many enzymes such as protein kinases and GTP-ases.) Such insensitivity implies a large fraction of neutral mutations among all mutations that affect enzyme concentration, which can thus evolve by neutral drift (Kacser and Burns 1981; Hartl et al. 1985; Nijhout and Berg 2003). Because robustness is not controlled independently from the core components of a system, it is not straightforward to disentangle buffering mechanisms that have been subject to natural selection from those that have not. This is a major challenge for future work.

Another open question is the extent to which trade-offs between different functions of a biological system influence the evolution of robustness. One might think, for example, that a gene regulatory network that needs to function in many different biological processes is more constrained in its evolution than a network deployed in only one process. For example, components of the Ras/MAP kinase pathway that are important in vulval fate induction also play a role in several other developmental decisions in *C. elegans*, as well as in olfaction and in response to pathogens (Sundaram 2006). A key question here is how the different selection pressures affecting pleiotropic mutations shape the evolution of robustness.

Evolutionary consequences of robustness

Mutational robustness causes an organism to tolerate changes. One immediate consequence is that for a robust trait, little genetic variation will be expressed as phenotypic variation. Natural selection, in turn, will be less effective in acting on the trait, at least in the short run, because the extent of phenotypic change that natural selection can cause strongly depends on phenotypically expressed genetic variation. Yet another immediate consequence is that *cryptic genetic variation* can accumulate, because neutral genetic variation accumulates faster than deleterious variation. The system can drift in neutral genotype space, and the larger the available neutral space, the more the system can drift. In other words, variation in an intermediate trait can accumulate without change in the robust final trait (Fig. 1C). In the face of environmental stressors that drive a system to the limit of its buffered range, such variation can become expressed at the level of the final phenotype. The vast majority of such expressed variation may be deleterious in these new conditions. However, a tiny fraction of it can harbor the seeds of new adaptations, which can change the evolutionary trajectory of an organism. Cryptic genetic variation may thus play two roles in phenotypic variation: allowing variation in intermediate phenotypes in the short term, and potential future phenotypic evolution in the final phenotype in the long term. Present controversies that remain to be experimentally addressed are two-fold: i. assessing whether such cryptic genetic variation evolves neutrally or under some kind of selection in the short term and ii. determining whether it may have a role in adaptation to new conditions in the long term.

Cryptic genetic variation is by definition difficult to detect. One way to uncover it is to experimentally drive the system out of its buffered range, using either environmental challenges such as heat shock or ether exposure as in the classical experiments by Waddington (Waddington 1942; Gibson and Hogness 1996), or mutations (Rutherford 2000; Gibson and Dworkin 2004). In

the latter case, the same mutation is introduced (usually by repeated crosses leading to introgression) into different wild genetic backgrounds. Cryptic variation in these wild genetic backgrounds can be detected as variation in mutational effects among the different backgrounds. For example, robustness properties of the vulva network ensures that three precursor cells adopt vulval fates in all wild isolates of *C. elegans*. However, cryptic variation between these wild isolates can be unmasked by displacing the system from the plateau of three induced cells. This is done by strongly reducing or increasing Ras pathway activity through mutations (Box 1, Fig. B3). Preliminary results suggest that the effect of Ras, Notch and Wnt pathway mutations does indeed vary significantly among different *C. elegans* wild genetic backgrounds (J. Milloz, I. Nuez and M.-A. F., unpublished). The robust vulva system thus accumulates cryptic variation, much like the robust cell fate patterning system of the *Drosophila* eye (Polaczyk et al. 1998). In the latter case, the genetic architecture of the cryptic variation is complex, involving variation at many loci and epistatic effects among them. Molecular variation at the EGF receptor locus contributes to a small but significant part of this variation (Dworkin et al. 2003). Understanding the genetic structure of cryptic genetic variation and the patterns of molecular evolution at the corresponding loci is an important current challenge (Gibson and Dworkin 2004).

An alternative way to detect cryptic variation is to turn to an "intermediate" phenotype, which may show variation between the tested conditions (Fig. 1A,C). One needs to clearly distinguish between the final output of the system, which is robust and invariant, and intermediate phenotypes that may be plastic in response to environmental variations and accumulate genetic variation (which is 'cryptic' when referring to the final phenotype). For example, the level of Ras pathway activity may vary between different wild *C. elegans* isolates without effect on the final cell fate pattern, either because the change is small and does not displace the population from the robust plateau, or because it is compensated by a change at another level (for example downstream in the same pathway). Using such an "intermediate" developmental phenotype, one can in principle reveal not only cryptic genetic variation, but also environmental or stochastic variation between individuals. Unraveling such variation remains an experimental challenge in robust developmental model systems.

In sum, we here discussed the concept of robustness, the nature of the perturbations to which biological systems can be robust, possible mechanistic and evolutionary causes of robustness, and the possible implications of robustness for evolution, all in the context of examples from the *C. elegans* vulva. These examples show that the challenges we face, even in a well-studied model system, greatly outnumber the insights we have. These challenges include to identify the prevalent mechanistic causes of robustness (redundancy or distributed robustness), to define the role of natural selection in their evolution, to identify the importance of trade-offs in multifunctional traits for the evolution of robustness, and to characterize the importance of cryptic variation for evolutionary innovation.

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Box 1. The *C. elegans* vulva, a robust developmental system

The vulva is the egg-laying and copulatory organ of the adult hermaphrodite of the nematode *C. elegans*. It is formed from a row of six competent vulva precursor cells, called P(3-8).p. During development, a reproducible spatial pattern of cell fates is formed within this row of six cells. Specifically, three of the cells adopt one of two vulval fates, either an inner vulval fate (1°, adopted by P6.p, blue) or an outer vulval fate (2°, adopted by P5.p and P7.p, red). The three remaining cells normally adopt non-vulval fates (3°, yellow), but are able to replace P(5-7).p. Formation of this fate pattern relies upon two kinds of intercellular signals. The first is an inductive signal from the uterine anchor cell (AC), which can act as a morphogen via the EGF-Ras-MAP kinase pathway. The second is a lateral signal that is transmitted between the Pn.p cells via the Notch pathway, which inhibits the 1° fate and activates the 2° fate (Sternberg 2005). In addition, a Wnt pathway (not shown) maintains the competence of the Pn.p cells in the second larval stage and cooperates redundantly with the Ras pathway in inducing vulval fates in the third larval stage (Eisenmann et al. 1998; Moghal et al. 2003).

Box 1 figures

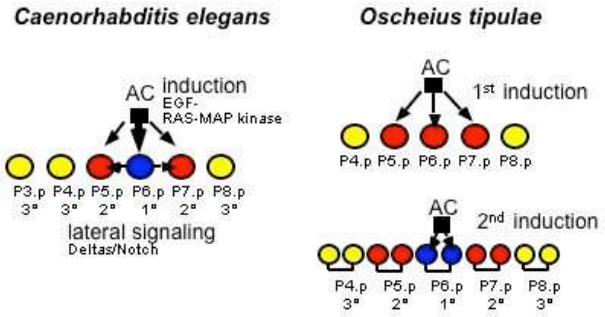

Fig. B1

Fig. B1. The vulval cell fate pattern is quasi-invariant among different species of the family Rhabditidae, including *C. elegans* and *Oscheius tipulae*. However, the mechanisms underlying cell fate patterning are different. One way to reveal this cryptic variation is to ablate the anchor cell to reveal its inductive action on Pn.p cells. In some species such as *Oscheius tipulae*, the anchor cell is required twice, first for the induction of 2° vulval fates, and then for the 1° vulval fates of P6.p daughter cells (Félix and Sternberg 1997). In *C. elegans*, the 1° fate of P6.p is specified earlier than in *O. tipulae*, and induces the 2° fates. In yet other species such as *Mesorhabditis* sp., removing the anchor cell has no effect on the development of the vulva cell fate pattern (Sommer and Sternberg 1994).

Box 1 figures

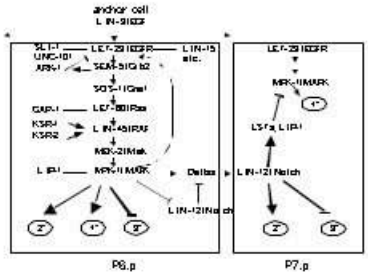

Fig. B2

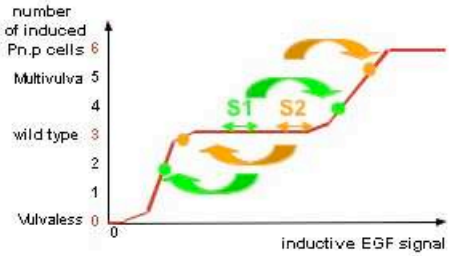

Fig. B3

Fig. B4

Fig. B2. The molecular network responsible for vulval cell fate specification shows buffering, redundancy, feedback loops and cross-talk at several levels of the intercellular signaling pathways (Sternberg 2005; Sundaram 2006). Here we show an outline of the network specifying P6.p (1°) and P7.p (2°) fates. After having received a signal from the epidermal growth factor receptor (EGFR) receptor via the Ras pathway, a mitogen-activated kinase (MAPK) activates vulval fate specification and transcription of Delta ligands. It also downregulates LIN-12/Notch in P6.p. Negative regulators (SLI-1, UNC-101, ARK-1, GAP-1, LIP-1) act at several positions along the Ras pathway. Single mutations in these genes have little effect on vulva development, but double mutants show a synthetic hyperinduced phenotype. In response to lateral signaling from P6.p through Notch, the neighboring cells P7.p and P5.p (not shown) upregulate the transcription of LIP-1, a phosphatase that inactivates MPK-1. In addition, they upregulate transcription of other lateral signaling targets (LSTs) that inhibit 1° fate specification. .

Fig. B3. Putative shape of the dose-response curve of the number of induced Pn.p cells (adopting a 2° or 1° fate) as a function of the amount of LIN-3/EGF. Robustness of the wild type pattern is visible as a plateau at 3 induced cells. This plateau is inferred from multiple experimental observations, especially that *lin-3/egf*, *let-23/egfr* or *let-60/ras* mutations are haplosufficient, and that single mutations in negative regulators are silent, yet double mutations have a multivulva phenotype. Animals of different genotypes (strains S1 and S2) may be located at different positions on this plateau. In addition, the location of any one genotype on the plateau may vary due to stochastic noise. Cryptic genetic variation among wild genotypes can be uncovered by driving the system from this plateau using perturbations (arrows), such as mutations in the signaling network or anchor cell ablations. Note that the number of induced cells is only a summary statistics that does not take into account the spatial fate pattern (2° and 1° fates).

Fig. B4. A rare developmental error in a *Caenorhabditis remanei* individual. As a result of this error P8.p adopts a vulval fate, as indicated by the ectopic vulval invagination on the right of the image.

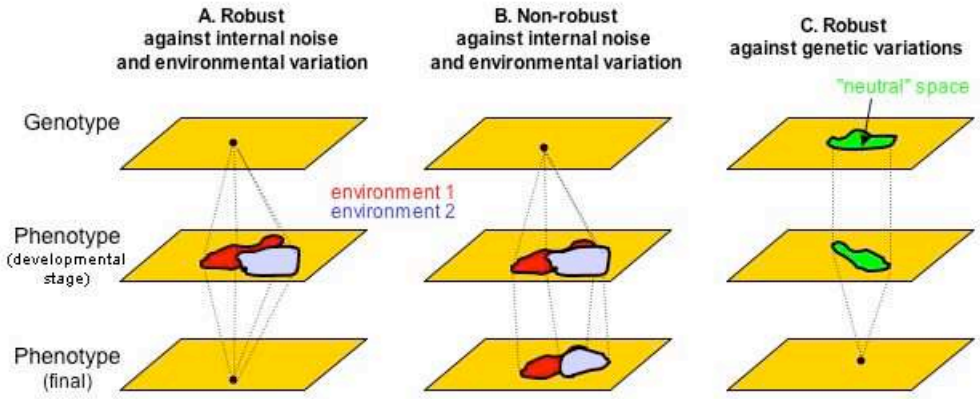

Figure 1

Figure 1. Robustness to stochastic noise, environmental change and genetic variations. Genotype and phenotype spaces are represented schematically in two dimensions. In (A) the final phenotype is robust to stochastic noise and environmental change. For any biological process, for example a developing system, the end product of the process may be robust whereas an intermediate trait (an intermediate metabolite concentration, a developmental stage in a multicellular organism, etc.) may not be robust. In (B) the system is not robust to the same perturbations. In (C) the system is robust to some genetic variation (green), thus allowing for cryptic variation to accumulate. A system that is robust to noise and a range of environmental variations (as in A) is likely to be robust to some genetic variation (as in C). The genotype space that produces the same final phenotype is "neutral" in this respect (and possibly also for fitness) yet intermediate phenotypes may display variation.

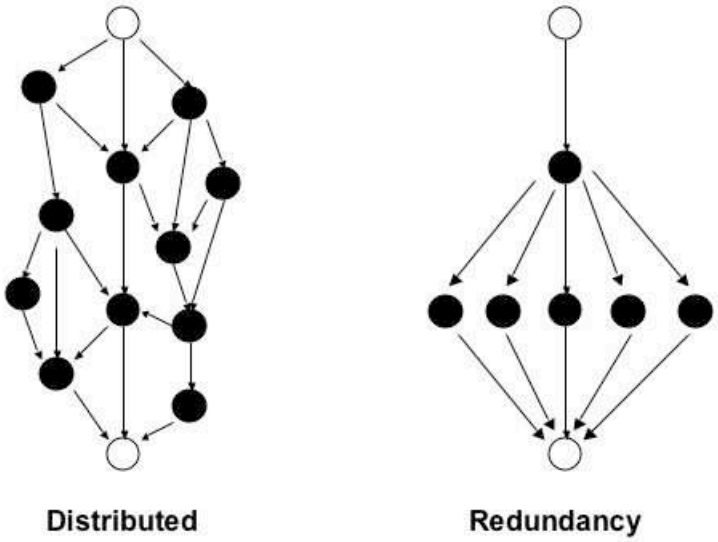

Figure 2

Figure 2. An illustration of distributed robustness versus redundancy. Both panels of the figure show a hypothetical signal transduction or metabolic pathway in which information about an upstream signal (upper white circles, e.g., the presence of a growth factor ligand) is communicated via a number of intermediate pathway components (black circles) to a downstream effector (lower white circles, e.g., a transcription factor). If a pathway like this shows distributed robustness (left), it is robust because the flow of information is distributed among several alternative paths, with no two parts performing the same function. In contrast, if robustness is achieved through redundancy (right), several components perform the same function.

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