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Phenotypic robustness can increase phenotypic variability after nongenetic perturbations in gene regulatory circuits

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Abstract

Nongenetic perturbations, such as environmental change or developmental noise, can induce novel phenotypes. If an induced phenotype appears recurrently and confers a fitness advantage, selection may promote its genetic stabilization. Nongenetic perturbations can thus initiate evolutionary innovation. Genetic variation that is not usually phenotypically visible may play an important role in this process. Populations under stabilizing selection on a phenotype that is robust to mutations can accumulate such variation. After nongenetic perturbations, this variation can produce new phenotypes. We here study the relationship between a phenotype's mutational robustness and a population's potential to generate novel phenotypic variation. To this end, we use a well-studied model of transcriptional regulation circuits that are important in many evolutionary innovations. We find that phenotypic robustness promotes phenotypic variability in response to nongenetic perturbations, but not in response to mutation. Our work suggests that nongenetic perturbations may initiate innovation more frequently in mutationally robust gene expression traits.

Introduction

Two main perspectives exist about the origin of evolutionary innovations. The orthodox ''genotype-first'' perspective emphasizes the role of mutations in the production of new phenotypes. In this perspective, mutations produce individuals with novel phenotypes whose frequency in a population may increase through natural selection. The heterodox ''phenotype-first'' perspective (West-Eberhard, 1989, 2003; Hall, 2001; Price *et al.*, 2003; Palmer, 2004; Newman *et al.*, 2006; Pigliucci *et al.*, 2006; Moczek, 2007; Gilbert & Epel, 2008) emphasizes the role of nongenetic perturbations, such as exposure to different temperatures, diets or biotic interactions. Nongenetic perturbations also comprise fluctuations in an organism's internal ''microenvironment'', such as gene activity changes caused by noisy gene expression (McAdams & Arkin, 1997; Elowitz *et al.*, 2002; Raj *et al.*, 2010).

The phenotype-first perspective is based on the observation that organisms often have highly plastic phenotypes. That is, the same genotype has the potential to produce different phenotypes depending on nongenetic influences. A nongenetic perturbation can thus trigger a plastic phenotypic response in some individuals of a population. If the resulting novel phenotype provides a benefit to its carrier, it facilitates survival. Subsequently, selection may increase the frequency of those genotypes that produce the beneficial phenotype and of new or already existing genetic variants that exaggerate, refine or ''stabilize'' this phenotype by making it independent of nongenetic factors. This process requires that the new phenotype appears recurrently and hence, that the nongenetic perturbations that induced it either persist for

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many generations (Kim, 2007; Griswold & Masel, 2009) or that they produce a persistent epigenetic effect (Sollars *et al.*, 2003; Gilbert & Epel, 2008). Waddington (1953) coined the term genetic assimilation for the stabilization of traits induced by nongenetic factors.

Increasing amounts of evidence suggest that traits induced by nongenetic factors are important for innovation (for dissenting opinions, see Orr, 1999; de Jong & Crozier, 2003). First, theoretical work shows that assimilation *can* occur under broad conditions (Wagner *et al.*, 1997; Rice, 1998; Masel, 2004; Ciliberti *et al.*, 2007b; Lande, 2009; Espinosa-Soto *et al.*, 2011). Second, laboratory evolution experiments show that assimilation *does* occur (Waddington, 1953, 1956; Rutherford & Lindquist, 1998; Suzuki & Nijhout, 2006; Eldar *et al.*, 2009). Third, studies in natural populations suggest that genetic assimilation of traits induced by nongenetic factors is not rare (Pigliucci & Murren, 2003; West-Eberhard, 2003; Palmer, 2004; Aubret & Shine, 2009). For example, taxa with genetically determined dextral or sinistral morphologies are frequently derived from taxa in which the direction of the asymmetry is not genetically fixed, but where it is a plastic response (Palmer, 1996, 2004). This occurs for many traits, such as the side on which the eye occurs in flat fishes (Pleuronectiformes) and the side of the larger first claw in decapods (Thalassinidea) (Palmer, 1996). Transitions like these indicate genetic assimilation of a direction of asymmetry originally induced by nonheritable factors. More generally, traits where fixed differences among closely related species are mirrored by plastic variation within populations are good candidates for genetic assimilation. For example, amphibian traits, such as gut morphology (Ledon-Rettig *et al.*, 2008), limb length and snout length (Gomez-Mestre & Buchholz, 2006), follow this pattern.

A system is robust to genetic or nongenetic perturbations if its phenotype does not change when perturbed. Mutational robustness and robustness to nongenetic perturbations are correlated with one another in many cases (Rutherford & Lindquist, 1998; Ancel & Fontana, 2000; Meiklejohn & Hartl, 2002; de Visser *et al.*, 2003; Ciliberti *et al.*, 2007b; Proulx *et al.*, 2007; Lehner, 2010), although exceptions exist (Cooper *et al.*, 2006; Masel & Siegal, 2009; Fraser & Schadt, 2010). The ability to produce evolutionary innovation is linked to the robustness of a biological system (Ancel & Fontana, 2000; Wagner, 2005; Ciliberti *et al.*, 2007a; Wagner, 2008b; Draghi & Wagner, 2009). For two reasons, robustness might seem to hamper innovation. First, a mutationally robust system produces less phenotypic variation in response to mutations. It may thus not facilitate the genotype-first scenario (Ciliberti *et al.*, 2007a; Draghi & Wagner, 2009). Second, a system robust to nongenetic factors shows little phenotypic plasticity. Thus, it may not support innovation under the phenotype-first scenario. However, the role of robustness in innovation is subtler than it seems. This becomes evident when one considers how genotypes and their phenotypes are organized in a space of genotypes.

Genotypes exist in a vast space of possible genotypes. Two genotypes are neighbours in this space if one can be transformed into the other by a single mutation. The distribution of phenotypes in genotype space shows some qualitative similarities for different kinds of systems, from RNA and protein molecules to metabolic networks and transcriptional regulation circuits. First, large sets of genotypes produce the same phenotype. Each of these sets can be traversed through single mutation steps that leave the phenotype unchanged. Such a set is also referred to as a neutral network or genotype network (Schuster *et al.*, 1994). Second, mutations of genotypes that lie in different regions of a genotype network can create very different novel phenotypes (Lipman & Wilbur, 1991; Schuster *et al.*, 1994; Schultes & Bartel, 2000; Ciliberti *et al.*, 2007a; Ferrada & Wagner, 2008; Wagner, 2008a; Rodrigues & Wagner, 2009).

To understand how mutational robustness relates to a system's ability to produce evolutionary innovations, it is useful to distinguish between the mutational robustness of a genotype and that of a phenotype. A genotype G_1 is mutationally more robust than another genotype G_2, if G_1 is more likely to maintain the same phenotype than G_2 in response to mutation. By extension, a phenotype P_1 is mutationally more robust than P_2 if the genotypes that produce P_1 preserve P_1, on average, more often than the genotypes adopting P_2 preserve P_2 in response to mutations. Surprisingly, mutational phenotypic robustness can facilitate the production of novel RNA structure phenotypes (Wagner, 2008a). The reason is that genotypes with a more robust phenotype form larger genotype networks and have, on average, more neighbours with the same phenotype. A population of such genotypes encounters relatively few deleterious mutations that would slow its diversification and spreading through genotype space (while preserving its phenotype). The resulting higher genotypic diversity translates into greater phenotypic variability in response to mutations, even though every single genotype may have access to fewer novel phenotypes (Wagner, 2008b).

This mechanism, although corroborated for RNA and protein structural phenotypes (Ferrada & Wagner, 2008; Wagner, 2008a), may not lead to increased phenotypic variability in all systems. The reason is that it depends on how many different and unique phenotypes the neighbourhood of different genotypes contains, and on how rapidly populations can spread through a genotype network. In other words, it depends on the organization of genotype networks in genotype space, which may differ among different system classes.

The above considerations pertain to phenotypic variability in response to mutations. As robustness to mutations and to nongenetic factors is often positively correlated (Rutherford & Lindquist, 1998; Ancel & Fontana, 2000; Meiklejohn & Hartl, 2002; de Visser

et al., 2003; Ciliberti *et al.*, 2007b; Lehner, 2010), one might think that phenotypic variability in response to nongenetic perturbations may behave similarly. However, we show that this is not necessarily so for transcriptional regulation circuits. Such circuits direct the production of specific gene activity patterns at particular times and places in the developing organism. Changes in the expression of their genes are involved in many evolutionary innovations (Davidson & Erwin, 2006; Shubin *et al.*, 2009). We study a generic computational model of transcriptional regulation in which the genotypes correspond to the cis-regulatory interactions in a transcriptional circuit. The phenotypes correspond to the gene activity pattern a circuit produces.

For this system, we have shown elsewhere that the organization of genotype space favours the evolution of new adaptive traits through a phenotype-first scenario. For example, we found that genotypes that can produce a new gene activity phenotype P after nongenetic perturbations have easy mutational access to genotypes where nongenetic perturbations are no longer necessary to produce P. Thus, new phenotypes induced by nongenetic perturbations can easily undergo genetic assimilation (Espinosa-Soto *et al.*, 2011). Because our previous results already show that the structure of gene circuit genotype space promotes assimilation, we here focus on the production of novel phenotypes. Specifically, we address how phenotypic robustness affects the potential of nongenetic perturbations and mutations to produce new phenotypes. We show that high phenotypic robustness to mutations increases the number of novel expression phenotypes that a population can produce in response to nongenetic perturbations. Thus, phenotypic robustness to mutation facilitates innovation under the phenotype-first scenario. It does so by allowing the accumulation of genetic variation that is not observed phenotypically under typical conditions, but that may be exposed after nongenetic perturbations (de Visser *et al.*, 2003; Masel, 2006; Masel & Siegal, 2009).

Methods

Model

The model represents a regulatory circuit of N genes, where each gene's activity is regulated by other genes in the circuit. The circuit's genotype is defined by a real-valued matrix $\mathbf{A} = (a_{ij})$, in which nonzero elements represent regulatory interactions between genes (Fig. 1a). An interaction ($a_{ij} \neq 0$) means that the activity of gene j can either have a positive ($a_{ij} > 0$) or a negative ($a_{ij} < 0$) effect on the activity of gene i. We use m to refer to the number of interactions in a given circuit and c to its interaction density, i.e. to the number of interactions m divided by the maximum possible number of interactions N^2. A vector $s_t = (s_t^{(1)}, \ldots, s_t^{(N)})$ describes the activity state of the circuit at time t.

The activity of the genes in the circuit changes according to the difference equation

$$s_{t+\tau}^{(i)} = \sigma\left[\sum_{j=1}^{N} a_{ij} s_t^{(j)}\right] \quad (1)$$

where $\sigma(x)$ equals -1 when $x < 0$, it equals 1 when $x > 0$, and it equals 0 when $x = 0$.

Variants of this model have proven useful for studying the evolution of robustness in gene regulatory circuits (Wagner, 1996; Siegal & Bergman, 2002; Ciliberti *et al.*, 2007b; Martin & Wagner, 2008), the effect of recombination on the production of negative epistasis (Azevedo *et al.*, 2006; Martin & Wagner, 2009), the evolution of modularity in gene circuits (Espinosa-Soto & Wagner, 2010) and the evolution of new gene activity patterns (Ciliberti *et al.*, 2007a; Kimbrell & Holt, 2007; Draghi & Wagner, 2009).

We consider asexual, haploid circuits that start their dynamics from a particular initial gene expression state s_0. One can view this initial state as being specified by factors external to the circuit, be they environmental factors, signals from adjacent cells, maternal regulators, or any genes ''upstream'' of the circuit. The phenotype is the stable (fixed-point) gene activity pattern s_∞ that a circuit attains when starting from s_0. Throughout our work, we disregard circuits that do not produce fixed-point equilibrium states or that produced phenotypes in which the activity of a gene is equal to zero (neither active nor inactive), as in previous research (Ciliberti *et al.*, 2007b). We consider circuits that attain the same s_∞ as equal with respect to their gene expression phenotype. Under this assumption, a mutation that transforms two such circuits into one another would be neutral with respect to this phenotype (Fig. 1b).

Determination of 1-mutant neighbourhoods

In several of our analyses, we explored properties of the circuits that differ from a reference circuit genotype G by one single mutation. In our approach, a mutation affects a single regulatory interaction between two genes. That is, it changes a single entry a_{ij} in the matrix $\mathbf{A}$ of G. Our underlying assumption is that mutations occur in regulatory regions, where mutations in one enhancer often have no effect in other enhancers (Prud'homme *et al.*, 2007; Wray, 2007). For simplicity, we also assume that every transcription factor binds to a different enhancer. We considered two kinds of single mutation for each entry a_{ij} in the matrix $\mathbf{A}$ of G: (i) if $a_{ij} = 0$, we considered one mutant where $a_{ij} < 0$, and another in which $a_{ij} > 0$; (ii) if $a_{ij} \neq 0$, we considered one mutant in which an interaction is lost ($a_{ij} = 0$), and another mutant in which we change the value of a_{ij} while keeping its sign unchanged. Among all the possible variants in the one-mutation neighbourhood of a circuit, we allowed exclusively those that maintained the number of interactions within an interval $[m_-, m_+]$, thus keeping interaction

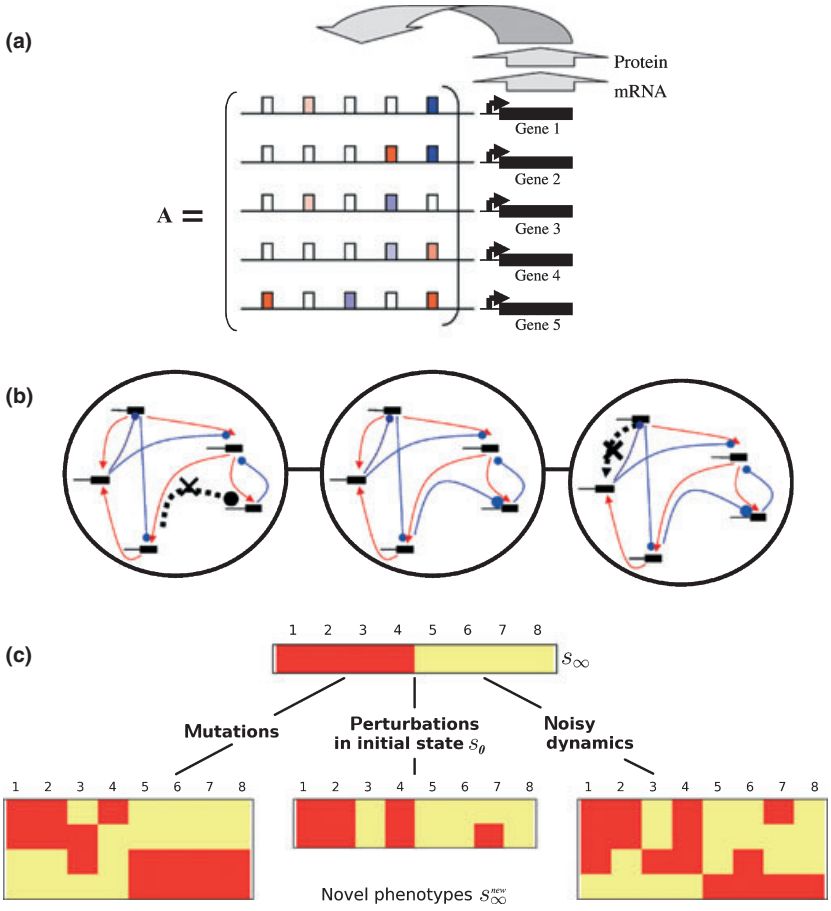

Fig. 1 Gene regulatory circuit model. (a) A gene regulatory circuit. Black bars indicate genes that encode proteins which regulate the activity of other genes in a hypothetical circuit. The regulatory interactions are described by a matrix $\mathbf{A} = (a_{ij})$. An interaction means that the activity of gene j can either have a positive ($a_{ij} > 0$, red rectangles) or a negative ($a_{ij} < 0$, blue rectangles) effect on the activity of gene i. (b) Gene circuits that differ in a single interaction are neighbours in genotype space. Each large circle surrounds a distinct gene regulatory circuit. Red arrows represent activating interactions, and blue lines represent repressing interactions between different genes (black rectangles). Dashed lines represent the interactions that are necessary to convert the indicated circuits into the middle circuit. (a) and (b) are modified with permission from Ciliberti *et al.* (2007b). (c) Example of novel phenotypes caused by three kinds of perturbations. A reference gene circuit produces phenotype s_∞, in which four genes are active (genes 5–8; yellow) and four genes are inactive (genes 1–4; red). In a one-mutation neighbourhood (all circuits that differ from the reference one by a single interaction), we find four phenotypes s_∞^{new} different from the original s_∞ (left). If we perturb the system state of the reference circuit without altering its genotype, other novel phenotypes are encountered (centre and right panels). The perturbations we used are either all single-gene perturbations in the initial condition s_0 (centre), or perturbations of the dynamical trajectory of the circuit (''noisy dynamics''; right).

density at a value close to c. Throughout this manuscript, $m_+ - m_- = 5$. Whenever a new nonzero value was required for a given a_{ij}, we chose a normally distributed ($N(0,1)$) pseudorandom number and forced its sign if needed. We defined the robustness to mutations of a genotype G as the fraction of G's 1-mutant neighbours that produce the same phenotype as G when their dynamics start from the initial state s_0. To assess the phenotypes that G can access through mutations, we registered and counted all the different phenotypes produced by the set of single mutant circuits that neighbour the reference circuit G. The approach is readily extended to entire populations. Whenever we applied it to entire populations, we counted phenotypes that occurred in the neighbourhood of two or more circuits only once.

Evolving populations

For the model we use, a given pair of initial and final expression states (s_0, s_∞^{opt}) is representative of all pairs with the same fraction d of individual genes' expression values that differ between s_0 and s_∞^{opt} (Ciliberti *et al.*, 2007b). For a prespecified d, we thus chose an arbitrary such pair and followed previously established procedures (Ciliberti *et al.*, 2007b) to identify a circuit genotype G

that is able to drive the system from s_0 to s_∞^{opt}. The regulatory interactions in the initial genotype G are real numbers sampled from a normal distribution with mean 0 and standard deviation 1, i.e. an N(0,1) distribution. After having identified one such genotype G, we created a population of 200 copies of it and subjected this population to repeated cycles (''generations'') of mutations (with a probability of mutation of $\mu = 0.5$ per circuit) and strong stabilizing selection on s_∞^{opt}. To mutate a circuit, we chose one of the circuit's 1-mutation neighbours at random.

Throughout, we interpret a circuit's ''fitness'' as a survival probability. We followed the regulatory dynamics of each gene circuit with s_0 as initial condition. We assigned circuits that attained an equilibrium state s_∞ that differed from s_∞^{opt} in the activity state of k $(0 \le k \le N)$ genes a fitness equal to $(1 - k/N)^5$, which ensures a steep decrease in survival probability even for small deviations from s_∞^{opt}. Thus, s_∞^{opt} represents a predetermined optimal gene expression state, upon which stabilizing selection acts. Each generation, we constructed a new population by sampling individuals with replacement from the previous generation, and subjecting copies of them to mutation with a probability μ. We kept each of these new individuals with a probability equal to its fitness and continued sampling until the newly generated population had 200 members. For all the populations we study, we let the initial population of identical genotypes evolve for 10^4 generations under selection for s_∞^{opt}, before collecting any simulation data. This allows the population to erase any traces of the initial genotype and to reach a plateau where phenotypic variability in response to either mutations or nongenetic perturbations varies little across generations.

In a distinct set of simulations, we explored how exposure to nongenetic perturbations in this preliminary period of stabilizing selection could affect a population's phenotypic variability. In these simulations, each circuit in a population was subject to nongenetic perturbations with probability β every generation. For each circuit undergoing nongenetic perturbations, we set the initial state of one of the circuit's genes picked at random to a random activity state (either −1 or 1).

We define the genotypic distance between two circuits as the minimum number of mutations needed to transform one circuit into the other, normalized by the maximally possible number of such mutations. The minimum number of mutations that set two genotypes apart is the number of differences between their matrices **A**. Whenever a pair of corresponding regulatory interactions a_{ij} are both different from zero and have opposite signs, we count the difference twice. The reason is that in our mutation procedure, changing the sign of a regulatory interaction a_{ij} requires at least two mutations. The maximal number of mutations between circuits with the same number of regulatory interactions is given by the sum of the number of interactions of both circuits.

Implementation of noise

We emulated the perturbations produced by noise in two complementary ways. First, we changed the activity state of single genes in the initial state s_0 for each gene in a circuit, and we determined the new phenotypes s_∞^{new} that resulted from such change.

Second, we perturbed the developmental dynamics (''noisy dynamics'') as follows: for each circuit in a population, we generated $5N$ dynamic trajectories, each of which started from s_0. For each of these trajectories, and for each step of the regulatory dynamics, we perturbed the activity of a randomly picked gene with a probability of 0.5. We then followed each trajectory until an activity pattern s had consecutively repeated itself and labelled this pattern as s_∞. We then counted the number of different fixed-point equilibrium states that each circuit could attain in these $5N$ trajectories.

Random sampling of genotypes in genotype networks

To sample properties of a given genotype network uniformly, we performed a random mutational walk restricted to this genotype network, that is, to circuits that attain a given s_∞^{opt} from the initial state s_0. We then examined properties of genotypes every n steps of this random walk, where n equalled 5 times the upper limit m_+ of the number of interactions in the circuit. This sporadic sampling serves to erase correlations in genotypes along this random walk.

Results

Genotype networks of gene expression phenotypes have different sizes

For our model, most or all genotypes that produce the same phenotype form large connected genotype networks (Ciliberti *et al.*, 2007a,b). The size of any one phenotype's genotype network depends only on the fraction d of genes whose expression state differs between the initial state s_0 and the steady-state activity phenotype s_∞^{opt} (Ciliberti *et al.*, 2007b). Specifically, phenotypes where these two states (regardless of their actual expression values) are more similar have larger genotype networks (Fig. S1). One can view regulatory circuits as devices that compute an expression state s_∞^{opt} from the initial state s_0. From this perspective, a larger number of gene expression differences between these states means that the computation becomes increasingly difficult, in the sense that fewer genotypes can perform it.

We examined in our model the relationship between the size of a phenotype P's genotype network and the robustness of circuits with this phenotype P to mutations. To this end, we pursued the following procedure for genotype networks of different sizes (different d). We

uniformly sampled 10^6 genotypes from a genotype network and determined their mean robustness to mutations, that is the mean fraction of their neighbours with the same phenotype. For all examined cases, the average mutational robustness (i.e. phenotypic robustness) is higher for genotypes on larger genotype networks when we control for the number N of genes in a circuit and the interaction density c (Fig. S2). Thus, phenotypic robustness to mutations increases with genotype network size, just as for RNA (Wagner, 2008a). Therefore, we can simply use $1 - d$ as a proxy for genotype network size and phenotypic robustness to mutations.

Phenotypic robustness to mutations facilitates phenotypic variability in response to noise

In this paper, we are concerned with the production of new steady-state gene expression patterns s_∞^{new} that are different from s_∞^{opt}. We refer to such activity patterns as new phenotypes. They could result from mutations that change regulatory interactions in a circuit. They could also result from *nongenetic* perturbations (Fig. 1c). We here consider two kinds of nongenetic perturbations, noise in a cell's internal environment and change in the organism's (external) environment. Both kinds can induce dramatic gene expression changes in organisms ranging from bacteria to metazoans (Elowitz *et al.*, 2002; Raj *et al.*, 2010; Snell-Rood *et al.*, 2010). We first focus on noise, which includes stochastic changes in protein or mRNA copy numbers in a cell, and which can cause phenotypic heterogeneity in clonal populations (McAdams & Arkin, 1997; Elowitz *et al.*, 2002; Raj *et al.*, 2010). Such noise may affect the activity or expression of circuit genes at a given time, which may alter a circuit's gene expression dynamics, and lead to a new steady-state activity pattern s_∞^{new}.

We emulated the perturbations produced by noise in two complementary ways. First, we perturbed the activity state of single genes in the initial state s_0. Second, we randomly perturbed the dynamic trajectory from s_0 to s_∞ (''noisy dynamics''; see details of both implementations in Methods).

We asked how the mutational robustness of a gene expression phenotype affects the number of new phenotypes that these two kinds of noise can produce in populations of evolving circuits. This number reflects the potential of a population to produce phenotypic variation through noise. We evolved populations of 200 circuits under stabilizing selection on a given gene expression state s_∞^{opt}, as described in Methods. We found that noise can produce more new and different phenotypes in populations evolving on large genotype networks. Figure 2 shows pertinent data for circuits with $N = 20$ genes and an interaction density $c \approx 0.2$. These observations also hold if we vary the numbers of genes and regulatory interactions in a circuit (Figs S3 and 4), with a single exception for perturbations in s_0 when the number of regulatory interactions is very low (Fig. S3d).

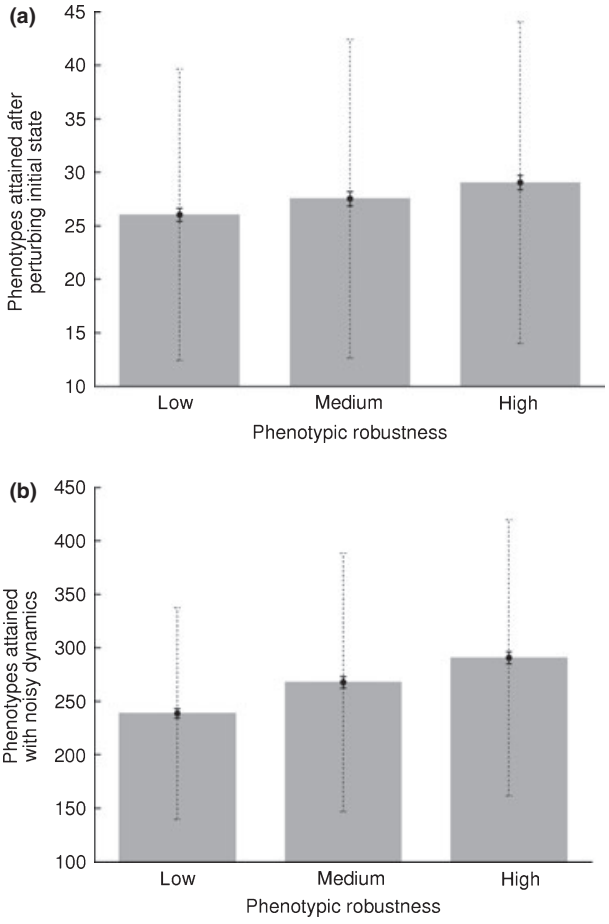

Fig. 2 High phenotypic robustness facilitates phenotypic variability in response to noise in gene expression. The distance d between the initial state s_0 and the optimal phenotype s_∞^{opt} is strongly associated with genotype network size and with phenotypic mutational robustness (''phenotypic robustness'') hereafter. 'High', 'medium' and 'low' correspond to expression phenotypes with high ($d = 0.1$), intermediate ($d = 0.25$) and low ($d = 0.5$) robustness. The figure shows results for $N = 20$ genes and a fraction $c \approx 0.2$ of nonzero regulatory interactions. Both panels show mean numbers of novel phenotypes averaged over 500 independent populations, for each level of robustness. The length of solid error bars denotes one standard error. The length of dashed bars indicates one standard deviation. The number of different new phenotypes that a population can access after perturbations of (a) single genes in the initial state s_0 or (b) a circuit's gene expression trajectory, increases with phenotypic robustness.

Populations with more robust phenotypes harbour more diverse genotypes

Increased genotypic diversity in populations evolving in large genotype networks might aid in producing increased phenotypic variability, as discussed in the

Introduction. We next asked whether this mechanism may apply to our system.

As a measure of a population's genotypic diversity, we estimated the mean pairwise circuit genetic distance, as well as its maximum, in each of 500 populations evolved under stabilizing selection on a phenotype s_∞^{opt} (see Methods). We did so for two classes of populations that differ in the robustness of their phenotypes and found that the mean genotypic distance is significantly higher for populations with a robust phenotype. The same holds also for the maximum genotypic distance. These observations are not sensitive to the number of genes and interactions in a circuit (Table S1). Thus, populations with a robust phenotype are genetically more diverse than populations with a less robust phenotype. These observations hint that the higher genetic diversity of populations with robust phenotypes may be exposed as phenotypic variability in response to noise.

Phenotypic robustness does not facilitate phenotypic variability caused by mutations

We next asked whether phenotypic robustness also facilitates phenotypic variability in response to mutations for the regulatory circuits we study. We again studied populations of circuits evolved under stabilizing selection on a phenotype s_∞^{opt}. In such populations, we determined the number of unique new gene activity phenotypes in the population's 1-mutation neighbourhood (see Methods). This number of unique phenotypes is a measure of the population's phenotypic variability in response to mutations. It thus reflects a population's potential to produce phenotypic variants through mutation.

We found that populations with a highly robust phenotype show lower phenotypic variability in response to mutations. This holds despite their somewhat higher genotypic diversity (Table S1). Figure 3 shows pertinent data for circuits with 20 genes and interaction density $c \approx 0.2$. The same behaviour holds for populations of circuits with different number of genes and different interaction densities (Fig. S5). In sum, robustness of a phenotype to mutations impairs phenotypic variability to mutation, as opposed to what we saw for variability in response to noise.

Our results suggest that a phenotype's mutational robustness promotes phenotypic variability in response to noise, but hinders such variability in response to mutations. This may seem surprising, because robustness to mutations increases with robustness to noise for individual circuits (Ciliberti *et al.*, 2007b). One might thus think that phenotypic variability also behaves similarly in response to these perturbations. However, robustness to mutations explains >25% of the variance in robustness to noise, as a new statistical analysis of our previously published data (Ciliberti *et al.*, 2007b) demonstrates (results not shown). Thus phenotypic variability in response to noise and to mutation is only weakly coupled.

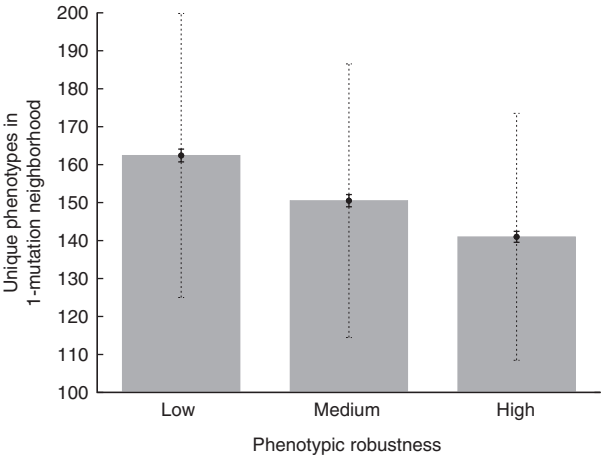

Fig. 3 High phenotypic robustness does not facilitate phenotypic variability in response to mutations without preceding environmental change. 'High', 'medium' and 'low' correspond to expression phenotypes with high ($d = 0.1$), intermediate ($d = 0.25$) and low ($d = 0.5$) robustness. The figure shows results for $N = 20$ genes and a fraction $c \approx 0.2$ of nonzero regulatory interactions. The panel shows the mean number of novel phenotypes averaged over 500 independent populations, at each level of robustness. The length of solid error bars denotes one standard error. The length of dashed bars indicates one standard deviation.

With these observations in mind, we analysed the phenotypic variability in response to noise and mutations of individual circuits in populations evolving on different genotype networks (Table S2). After having obtained these data, we compared the mean number of new phenotypes that mutations or noise could produce from circuits in populations with different levels of phenotypic robustness (Table S3). We found that phenotypic variability in response to gene expression noise decreases less with phenotypic robustness to mutations than phenotypic variability to mutations (Table S3). It may even increase with phenotypic robustness. These observations suggest that the increased genotypic diversity attained on larger genotype networks is insufficient to compensate for the reduction in variability in response to mutations. It is, however, sufficient to compensate for the smaller (or null) reduction in phenotypic variability in response to noise in gene expression.

Phenotypic robustness increases phenotypic variability after environmental change

Thus far, we focused mostly on phenotypic variability in response to small, random nongenetic perturbations, such as single gene expression perturbations along a gene expression trajectory. For such perturbations, we found that phenotypic robustness favours phenotypic variability. We now turn to the question of what happens when

a whole population is subject to the same nongenetic perturbation. In nature, this may occur because of environmental change outside the organism or colonization of a new habitat.

The environment can have two different roles in this context. The first is an inducing role, where the environment acts as an ''agent of development'' (West-Eberhard, 1989). In this role, it affects the phenotype produced from a genotype. In many cases, environmentally induced phenotypic change is linked to major changes in gene expression (Snell-Rood *et al.*, 2010). The second role is an evaluating role, where the environment acts as an ''agent of selection'' (West-Eberhard, 1989). In anthropomorphic terms, the environment in this role distinguishes well-adapted from poorly adapted phenotypes.

Conveniently, our model allows us to study these roles independently. We model a change in the environment's evaluation role as a change in the identity of the optimal phenotype s_∞^{opt}, for all circuits in the population. We model a change in the environment's inducing role as a change in the initial state s_0 in the whole population. Such a change could occur, for example, through a signaling pathway that detects an environmental change and that affects genes upstream of the circuit. Put differently, changes in s_0 reflect the environment's effect on phenotype production, whereas changes in s_∞^{opt} affect the survival probability of individuals, without inducing novel phenotypes. We note that other factors, such as mutations in upstream genes, might also lead to changes in s_0. Any one such change, however, would initially affect only one individual in a population and not the whole population at the same time.

We first asked how an environmentally induced change in the initial gene activity pattern s_0 affects the number of different actual phenotypes that a population displays. We note that our populations may contain a few individuals with phenotypes different from the optimal phenotype s_∞^{opt}. The reason is that, in contrast to previous formulations (Ciliberti *et al.*, 2007b), we here represent fitness as a continuous variable that depends on the similarity of a circuit's phenotype s_∞ to s_∞^{opt} (see Methods). We started out with a population evolved under stabilizing selection on an optimal expression phenotype s_∞^{opt} and a given gene activity pattern s_0^a as initial condition. We then counted the number of phenotypes in the population and compared it with the number of different phenotypes that the same population displays when s_0^a is replaced by a random gene activity pattern s_0^b as an initial condition. We found that phenotypic diversity increases after substitution of s_0^a with s_0^b (Figs 4 and S6). In addition, the magnitude of this increment increases with phenotypic robustness (Fig. 4 and Table S4). This last observation is generally not sensitive to the number of genes and regulatory interactions in a circuit (Fig. S6). The single exception to these observations was circuits of very low interaction density ($N = 20$; $c \approx 0.1$; Fig. S6d)

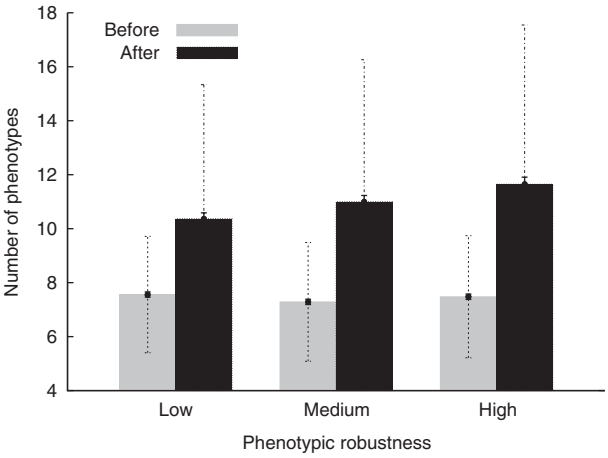

Fig. 4 High phenotypic robustness increases phenotypic diversity in populations of gene circuits after environmental change. The number of different phenotypes that populations display increases after changing the initial expression state s_0. Such an increase is greater for populations with mutationally more robust phenotypes. The figure shows results for $N = 20$ genes and a fraction $c \approx 0.2$ of nonzero regulatory interactions. The panel shows the mean number of observed phenotypes averaged over 500 independent populations, for each level of robustness. The length of solid error bars denotes one standard error. The length of dashed bars indicates one standard deviation.

that also show other nontypical behaviors (Ciliberti *et al.*, 2007b). Our results suggest that, after environmental change, observable phenotypic diversity increases to a larger extent in populations with a robust phenotype. We note that because the identity of s_∞ does not affect the production of phenotypes, but only their viability, it is not appropriate to carry out an analogous analysis for changes in s_∞^{opt}.

In earlier sections, we have shown that phenotypic robustness impedes phenotypic variability after mutations in populations evolving in a constant environment (Fig. 3). We next asked whether this also holds after a change in the inducing role of the environment. We started out, as in our last analysis, with populations of circuits evolved under stabilizing selection on an optimal expression phenotype s_∞^{opt} and with a given gene activity pattern s_0^a as initial condition. Then, we changed the initial condition s_0^a for all the circuits to a new random initial condition s_0^b and allowed evolution to proceed. Before and after this change, we recorded the number of different phenotypes accessible from the population through mutations. Under the new condition, the population effectively searches genotype space for optimal phenotypes. During this search, many variant circuits may not survive and be passed on to subsequent generations. Here, however, we do not focus on this search but on the effect on a population's potential to produce phenotypic variation through mutation immediately after environmental change.

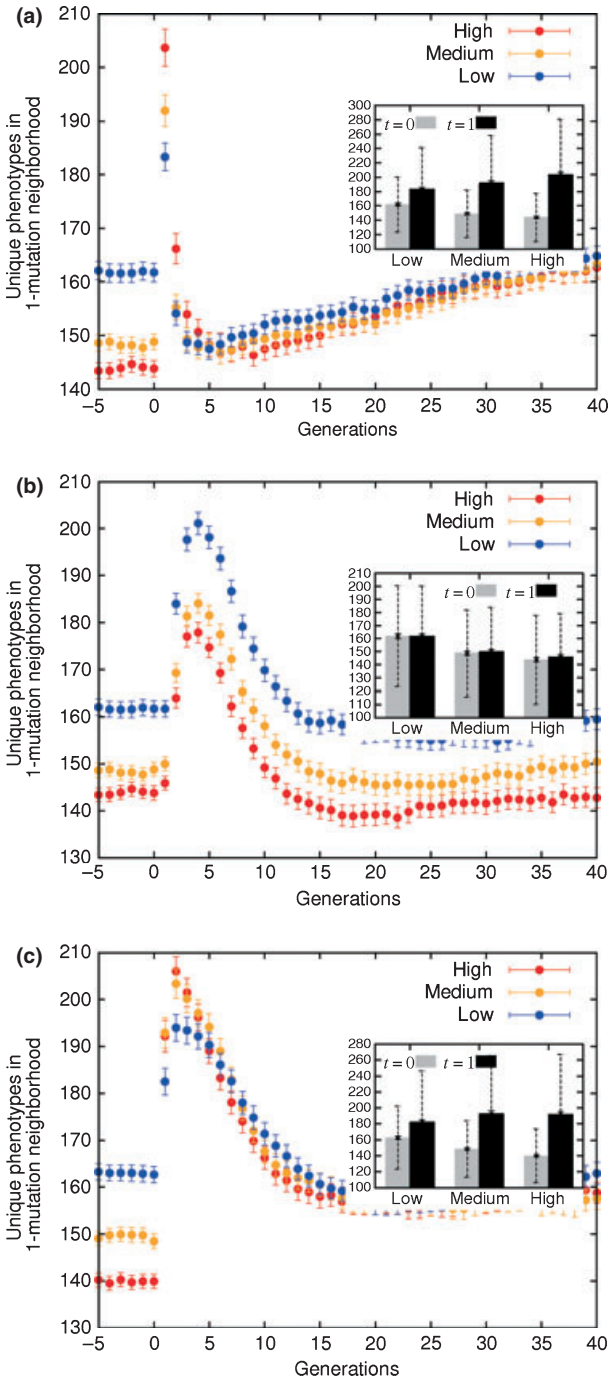

Fig. 5 High phenotypic robustness allows mutational access to more phenotypes after an environmental change produces novel phenotypes. The figure shows the number of different phenotypes in the 1-mutant neighbourhood of a population, for three different scenarios of environmental change at generation $t = 1$. The insets show the number of phenotypes accessible through mutation immediately before ($t = 0$) and immediately after ($t = 1$) environmental change. The figure shows results for $N = 20$ genes and a fraction $c \approx 0.2$ of nonzero regulatory interactions. 'High', 'medium' and 'low' correspond to expression phenotypes with high ($d = 0.1$), intermediate ($d = 0.25$) and low ($d = 0.5$) phenotypic robustness. Data are mean values averaged across 500 independent simulations for each level of robustness. The length of solid error bars denotes one standard error. The length of dashed bars (in the insets) indicates one standard deviation. (a) The initial state s_0 changes. (b) The identity of the optimal phenotype s_∞^{opt} changes. (c) The original pair (s_0^a, $s_\infty^{opt,a}$) changes to a new pair (s_0^b, $s_\infty^{opt,b}$).

Before environmental change, populations with a robust phenotype have access to fewer phenotypic variants, just as in our previous observations (Fig. 3). Immediately after environmental change (at $t = 1$), the number of new phenotypes accessible through mutations increases, in a burst, in all populations. Importantly, this increase is higher in populations with a robust phenotype (Figs 5a and S7). This means that phenotypic robustness facilitates the phenotypic variability caused by mutations, but only after environmental change. As in our analysis above, the only exception occurs when interaction density is very low (Fig. S7d).

We next asked whether the evaluation role of the environment has similar effects on mutational access to new phenotypes. To this end, we repeated the above analysis, but replaced, at $t = 1$, the optimal phenotype $s_\infty^{opt,a}$ by a randomly chosen optimal $s_\infty^{opt,b}$ (without changing s_0). We also observed a transient, albeit delayed and more gradual increase in the number of phenotypes that are mutationally accessible. In this case, phenotypic variability after environmental change is lower for populations with a robust phenotype (Figs 5b and S8). Thus the inductive role of environment, but not its evaluative role, causes higher phenotypic variability in populations with robust phenotypes.

An open question is how mutation-accessible phenotypic variability changes when both the inductive and the evaluation roles of the environment change. This question is important because a change in the inductive role favours phenotypic variability to a larger extent in populations with a robust phenotype, whereas a change in the evaluative role particularly favors variability in populations with less robust phenotypes. Thus, a combination of both effects could result in a negligible effect of phenotypic robustness on variability after environmental change. To answer this question, we repeated our analysis from the previous paragraph, but replaced the original *pair* of states (s_0^a, $s_\infty^{opt,a}$) with a new pair (s_0^b, $s_\infty^{opt,b}$), such that the distance d between s_0 and s_∞^{opt} was the same for both pairs.

In this new analysis, populations evolving on a large genotype network show greater phenotypic variability in response to mutations immediately after this change (Fig. 5c). These differences are statistically highly significant (Table S5). The same observations hold for circuits of different sizes and different interaction densities (Fig. S9 and Table S5). As in our analysis above, the

only exception occurs when the interaction density is very low (Fig. S9d). These observations imply that the inductive role dominates in its immediate effect on phenotypic variability when both roles of the environment change. In sum, populations with a robust phenotype have mutational access to more phenotypic variants after environmental change. This increased access is caused by the inductive role of the environment, that is by the new phenotypes that a new environment can bring forth.

The effect of phenotypic robustness on variability also occurs after long-term stabilizing selection in the presence of nongenetic perturbations

In all the simulation results that we report earlier, we evolved populations under stabilizing selection in the presence of mutations only, before collecting any data. One may ask what happens in a different scenario, when these populations are also subject to recurrent nongenetic perturbations. Long periods of stabilizing selection in the presence of nongenetic perturbations may promote the accumulation of circuit genotypes that only rarely produce (maladaptive) new phenotypes after nongenetic perturbations. The resulting differences in the distribution of circuits in genotype space may alter the positive effect of phenotypic robustness on variability. We next determined whether this is the case.

To this end, we allowed populations of circuits to evolve under stabilizing selection on a given phenotype for 10^4 generations. Throughout this period, small random nongenetic perturbations altered the dynamics of some fraction of the circuits in the populations (see Methods). We found that in these populations, phenotypic robustness still has a positive effect on variability caused by noise (Fig. S10). The effect of phenotypic robustness on variability is clearly visible as long as the probability β of a circuit undergoing nongenetic perturbations throughout the period of stabilizing selection is not too high, that is when populations are evolved under recurrent but not too *intense* noise (Fig. S10). Moreover, populations with a robust phenotype still form more *novel* phenotypic variants after a change in the environment's inducing role (not shown).

The distribution of a population's circuits in genotype space also depends on whether or not genotypes with a high mutational robustness are favoured in evolution. This difference may also alter the relationship between phenotypic robustness and variability that we observe. Stabilizing selection favours genotypes with high mutational robustness if the product of population size M and mutation rate μ is much greater than one (van Nimwegen *et al.*, 1999). All of our analyses above pertained to this case. However, we also studied the opposite extreme, in which selection cannot lead to the accumulation of genotypes with high mutational robustness: a single individual (a population of size one) exploring a genotype network through random mutations. In this analysis, we focused on the *cumulative* number of new phenotypes that this ''population'' can explore as a result of noise and mutation. We found that with increasing phenotypic robustness, the cumulative number of phenotypes accessible through noise increases (Fig. S11a, b), but cumulative phenotypic variability after mutation decreases (Fig. S11c). Thus, whether selection can or cannot increase genotypic mutational robustness does not affect qualitatively the effect of phenotypic robustness on phenotypic variability after nongenetic perturbations and mutations.

Discussion

If nongenetic change is to be causally involved in evolutionary innovation, it needs to generate novel, potentially beneficial phenotypes. Genetic assimilation can then stabilize one such beneficial phenotype, if the nongenetic perturbations that induced it either appear recurrently (Kim, 2007; Griswold & Masel, 2009), or if they have effects that persist for several generations (Sollars *et al.*, 2003; Gilbert & Epel, 2008). Previously, we studied the structure of the genotype space associated with a model of gene regulatory circuits. We showed that this structure facilitates the genetic assimilation of adaptive phenotypes that initially appear only after nongenetic perturbations (Espinosa-Soto *et al.*, 2011). Therefore, we here left genetic assimilation aside and concentrated on earlier evolutionary events, namely the origins of novel traits. Specifically, we used the same model of gene regulation to ask whether the robustness of an existing phenotype and nongenetic change can facilitate the origin of new phenotypes.

We focused on gene regulatory circuits for two reasons. First, many important adaptations appear at the level of gene regulation (Davidson & Erwin, 2006; Prud'homme *et al.*, 2007; Shubin *et al.*, 2009), and second, empirical evidence is especially supportive of the phenotype-first scenario for morphological and developmental traits [e.g. directional asymmetry in diverse taxa (Palmer, 2004), head size in australian tiger snakes (Aubret & Shine, 2009), or amphibian limb or gut morphology (Gomez-Mestre & Buchholz, 2006; Ledon-Rettig *et al.*, 2008)]. The production of these traits depends to a great extent on the dynamics of gene regulation. In the generic model of transcriptional regulation circuitry that we examined, the relationship between genotypes (patterns of regulatory interactions) and phenotypes (gene activity or expression patterns) is well studied (Wagner, 1996; Ciliberti *et al.*, 2007a,b; Martin & Wagner, 2008, 2009). In this model, we can use the size of a phenotype's genotype network as a proxy for a phenotype's robustness to mutations.

We analysed the potential of different kinds of perturbations to generate phenotypic variation in populations that were subject to stabilizing selection for many

generations. Such populations accumulate genetic variation that is not phenotypically visible (Gibson & Wagner, 2000). New genetic perturbations, such as gene knockout mutations, have the potential to expose this hidden genetic variation (Bergman & Siegal, 2003; Tirosh *et al.*, 2010) and facilitate adaptation to a new optimum (Bergman & Siegal, 2003). In addition, nongenetic perturbations may convert the accumulated genetic variation into evolutionarily meaningful phenotypic variation (Schmalhausen, 1949; West-Eberhard, 2003; Hermisson & Wagner, 2004).

We broadly distinguished two kinds of nongenetic perturbations. The first corresponds to fluctuations in a gene circuit's microenvironment that have important effects on gene expression phenotypes (McAdams & Arkin, 1997; Elowitz *et al.*, 2002; Raj *et al.*, 2010). The second kind comprises changes in the (macro)environment external to an organism. For brevity, we refer to these kinds of change as noise and environmental change.

We first found that phenotypic mutational robustness increases phenotypic variability of populations in response to noise but not in response to mutations. This last finding differs from observations for RNA secondary structure, where phenotypic robustness facilitates the mutational access to phenotypic variants (Wagner, 2008a). The reason stems from differences in the organization of genotype space for these two system classes, i.e. in the arrangement of different genotypes and genotype networks in genotype space (Ancel & Fontana, 2000; Ciliberti *et al.*, 2007a; Wagner, 2008a; Espinosa-Soto *et al.*, 2011). For example, nongenetic perturbations do not favour increased access to new phenotypes for RNA structures (Ancel & Fontana, 2000), but they do so for gene activity phenotypes (Espinosa-Soto *et al.*, 2011). A recent mathematical model (Draghi *et al.*, 2010) shows that mutational access to new phenotypes can depend on the organization of genotype space and on details of a population's evolutionary dynamics. It shows that phenotypic variability may vary nonmonotonically with mutational robustness, reaching a peak at intermediate values of robustness. Because multiple factors can affect phenotypic variability, it is not surprising that in system classes as different as molecules and regulatory circuits, robustness affects variability in different ways.

Next, we showed that environmental change, besides increasing observable phenotypic variation, transiently increases phenotypic variability caused by mutations. Because mutational access to most novel variants is only possible in the new environment, these variants can be considered environmentally induced phenotypes, supporting the phenotype-first scenario. Importantly, this increase in phenotypic variability is higher in populations that had a more robust phenotype before environmental change.

In sum, we found a positive effect of phenotypic robustness on phenotypic variability after nongenetic perturbations. This positive effect is not sensitive to the magnitude of nongenetic perturbations. Phenotypic robustness favours phenotypic variability after single-gene perturbations in the initial gene activity state, which is the smallest possible nongenetic perturbation in our model (Fig. 2a). It also favours phenotypic variability after replacing the initial state by a completely new random gene activity state (Figs 4 and 5a). In contrast, populations with robust gene expression phenotypes are phenotypically less variable in response to mutations. Thus, a mechanism that relies exclusively on mutation to produce novel phenotypes becomes less important for innovation as a phenotype's robustness increases. Our results suggest that plasticity-mediated innovation may be especially important for gene expression traits with high mutational robustness. Our work thus hints under what circumstances the phenotype-first scenario is more likely to underlie the origin of new traits. In this regard, we note that nongenetic induction of novel traits is not expected exclusively for gene circuits with (mutationally) robust phenotypes. We observe a general increase in phenotypic variability after environmental change (Figs 4 and 5). This increase is just especially marked for robust phenotypes.

We note that the effects of phenotypic robustness on variability in response to nongenetic perturbations or mutations that we observe do not change qualitatively when populations evolve in the presence or absence of indirect selection for mutational robustness (Fig. S11). Thus, the manner in which phenotypic robustness affects variability in response to perturbation may be specific to the kind of perturbation.

Our observations also hold when populations evolve under stabilizing selection and in the presence of recurrent but mild nongenetic perturbations (Fig. S10). However, the positive effect of phenotypic robustness on phenotypic variability may not occur in populations that have evolved under intense and recurrent nongenetic perturbations. Whether this observation undermines in a significant manner the generality of our conclusions is an open question. The answer will not only depend on how often real gene circuits are subject to nongenetic perturbations along their evolution, but also on how frequently such perturbations change the activity of genes in a given gene regulatory circuit. Additional factors, such as the sensitivity to perturbation of gene regulatory circuits ''upstream'' of the circuit under study, will also affect whether the effect that we describe will occur for a specific circuit.

We emphasize that our conclusions are not in conflict with previous theoretical (e.g. Bergman & Siegal, 2003; Hermisson & Wagner, 2004) and experimental (Tirosh *et al.*, 2010) research, which shows that major genetic alterations can expose hidden genetic variation. Our results merely suggest that phenotypic robustness does not favour phenotypic variability caused by mutations, not that mutations do not increase variability. Also, our results

are consistent with the observation that mutations can cause increased sensitivity to nongenetic perturbations (Levy & Siegal, 2008). In fact, we show that a nongenetic perturbation such as an environmental change can enhance the potential of mutation to generate variation (Figs 5a,c). This last observation is also consistent with previous work on niche evolution, which shows that nongenetic perturbations can accelerate adaptation to a new environment (Kimbrell & Holt, 2007).

The general increase in phenotypic variability we observe is consistent with many empirical observations on phenotypic variation that is conditional on the environment. For example, severe environments enhance phenotypic differences among fruit fly strains (Kondrashov & Houle, 1994), and a temperature rise caused by a lack of shade increases the frequency of abnormal morphologies in fruit flies (Roberts & Feder, 1999). Moreover, population genetic theory predicts that the release of hidden genetic variation after environmental change should be very common (Hermisson & Wagner, 2004).

In conclusion, our observations suggest that phenotypic robustness to mutations can play a positive role in phenotypic variability after nongenetic perturbations. To see this, one needs to study the role of population level processes, as we did. We caution that we made our observation in the context of a specific model of transcriptional regulation circuits. The gene expression phenotypes of such circuits play central roles in many evolutionary innovations (Davidson & Erwin, 2006; Shubin *et al.*, 2009). However, phenotypes may be distributed differently in genotype space in other classes of biological systems. Whether our observations hold in these systems remains to be seen.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Figure S1 The fraction of genotypes ($f(dN)$) that produce a phenotype decreases with dN.
Figure S2 Phenotypic mutational robustness increases with genotype network size.
Figure S3 High phenotypic robustness facilitates phenotypic variability in response to gene expression noise in s_0.
Figure S4 High phenotypic robustness facilitates phenotypic variability in response to noisy gene expression dynamics.
Figure S5 High phenotypic robustness does not facilitate phenotypic variability in response to mutations without preceding environmental change.
Figure S6 High phenotypic robustness increases phenotypic diversity in populations of gene circuits after environmental change.
Figure S7 High phenotypic robustness allows mutational access to more phenotypes after an alteration of the environment's inducing role.
Figure S8 High phenotypic robustness does not facilitate mutational access to more phenotypes after an alteration of the environment's evaluating role.
Figure S9 High phenotypic robustness allows mutational access to more phenotypes after an alteration of both the inducing and evaluating role of the environment.
Figure S10 Phenotypic variability in response to gene expression noise increases with phenotypic robustness in populations evolved under stabilizing selection and recurrent nongenetic perturbations.
Figure S11 Cumulative number of new phenotypes that a single gene circuit encounters after iterative rounds of mutation that preserve its phenotype.
Table S1 The mean and maximum genetic distance within a population increases with phenotypic robustness according to a Mann–Whitney U test.
Table S2 Mean number of new phenotypes accessible from individual genotypes in populations evolving in different genotype networks.
Table S3 Relative phenotypic variability of genotypes in large genotype networks.
Table S4 The increase in the number of different phenotypes after changing s_0 is larger in circuits with robust phenotypes according to a Mann–Whitney U test.
Table S5 The increase in the number of accessible new phenotypes is larger in circuits with robust phenotypes, according to a Mann–Whitney U test.

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