

Evolutionary constraints in variable environments, from proteins to networks

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Environmental changes can not only trigger a regulatory response, but also impose evolutionary pressures that can modify the underlying regulatory network. Here, we review recent approaches that are beginning to disentangle this complex interplay between regulatory and evolutionary responses. Systematic genetic reconstructions have shown how evolutionary constraints arise from epistatic interactions between mutations in fixed environments. This approach is now being extended to more complex environments and systems. The first results suggest that epistasis is affected dramatically by environmental changes and, hence, can profoundly affect the course of evolution. Thus, external environments not only define the selection of favored phenotypes, but also affect the internal constraints that can limit the evolution of these phenotypes. These findings also raise new questions relating to the conditions for evolutionary transitions and the evolutionary potential of regulatory networks.

Epistasis in variable environments

Evolutionary adaptation is commonly thought of in terms of two distinct factors. On the one hand, external selective environments drive evolution to particular favored phenotypes, whereas, on the other hand, internal organismal constraints limit access to these phenotypes. Generally, evolution may be limited by physicochemical constraints [1] or by genetic exigencies [2], for instance when rare combinations of mutations are required for a functional change. In laboratory experiments, selection and constraint have been quantified for environments and phenotypes that are constant in time [3–7]. In comparison, little is known about selection and constraint in variable environments. The effects of environmental variability could be significant: different environments may not only favor different phenotypes, but also give rise to different evolutionary constraints and, hence, blur the line between the external and internal factors that determine evolutionary adaptation.

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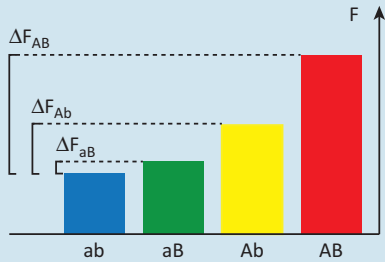
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These issues are of general relevance given the variable character of natural environments. They are important for regulatory systems in particular. Regulatory systems may well experience selection and evolve in constant conditions, but their ability to respond to environmental changes is logically considered to be shaped by a history of selection in changing environments [8]. However, the mechanisms of regulatory evolution in variable environments remain incompletely understood, despite detailed insights into function [9–11] and sequence evolution [12–16]. Elucidating these questions will be central to understanding how the complex regulatory circuitries of cells have evolved, may offer routes to engineer synthetic regulatory functions, and provide new perspectives on the function of regulatory networks.

At the most elementary level, genetic constraints in constant environments can be expressed in terms of the interaction between two mutations, which is commonly referred to as epistasis (Table 1). For instance, a reconstruction of neighboring genotypes of the protein β -lactamase revealed that mutating a particular residue could increase resistance to antibiotics, but only if a second residue was mutated first, otherwise the resistance decreased [4,17]. Such sign–epistatic interactions [5–7] can result from the highly integrated nature of molecular structures [18] and the interplay between protein stability and catalytic activity [19]. Sign epistasis affects selection, because fitness-increasing mutations are more readily fixed than neutral or fitness-decreasing mutations. In particular, the mutations will then be fixed in a specific order. Thus, sign–epistatic interactions between functionally important mutations constrain the number of mutational pathways accessible by positive selection. By contrast, forms of epistasis without changes in the sign of the effect, such as positive or negative epistasis (Table 1), do not have such drastic effects on selection, although they do provide important insights into functional relations.

The number of paths accessible by positive selection may also reduce to zero. Such a lack of available positively selected mutations could underlie cases of prolonged evolutionary stasis, and can be visualized as entrapment on a suboptimal fitness peak in genotype space [2]. Escape from such evolutionary stasis does remain possible in principle, for instance when multiple mutations are jointly fixed [4,20], or when population expansion limits selection and maintains less fit phenotypes [6], although at much reduced

Table 1. Types of epistasis

| Type of epistasis | Evolutionary consequences |
|---|--|
| No epistasis: $\Delta F_{AB} = \Delta F_{Ab} + \Delta F_{aB}$ | Both paths from ab to AB are accessible by positive selection. |
| Magnitude epistasis: $\Delta F_{Ab}, \Delta F_{aB} > 0$ | Positive: $\Delta F_{AB} > \Delta F_{Ab} + \Delta F_{aB}$ |
| | Negative: $\Delta F_{AB} < \Delta F_{Ab} + \Delta F_{aB}$ |
| Sign epistasis: $\Delta F_{Ab} < 0$ XOR $\Delta F_{aB} < 0$ | One path is accessible by positive selection, whereas the other is not; hence, a particular order of mutations is favored. |
| Reciprocal sign epistasis: $\Delta F_{Ab} < 0$ AND $\Delta F_{aB} < 0$ | Both paths from ab to AB are inaccessible by positive selection; this is a necessary condition for the existence of multiple local optima. |
|  | |
| <p>Figure T1. Types of epistasis in constant environments and their evolutionary consequences. Between genotypes ab and the fitness optimum AB, two mutational paths are possible: via Ab and via aB. ΔF_{Ab}, ΔF_{aB}, and ΔF_{AB} are the fitness changes relative to the fitness of ab. We note that, because neutral mutations are not positively selected, conditionally neutral mutations ($\Delta F_{Ab} = 0$ OR $\Delta F_{aB} = 0$) can be considered to exhibit (reciprocal) sign epistasis rather than magnitude epistasis.</p> | |

probability. It has been shown on theoretical grounds that, for systems to display this more severe genetic constraint, they must exhibit reciprocal sign–epistatic interactions (Table 1) [21]. In this case, two mutations are jointly beneficial but each individually deleterious. Such interactions have been observed in the regulator *MSN Three Homolog 1* (MTH1) and transporters hexose transporter 6 and 7 (HXT6 and HXT7) of the yeast glucose utilization pathway [22], among other systems [23].

An emerging question is how epistasis and constraint are affected by environmental variability. Not only is the natural environment intrinsically variable, but the effects of mutations are also often found to depend strongly on the environment. For instance, the change in growth rate for different *Escherichia coli* Tn10 transposon mutants was found to depend on not only the genetic background, but also the type of growth media used [24]. Such interactions between genetic and environmental changes are pervasive in biological systems [25–30]. These observations raise the question of how epistasis itself is impacted by environmental variability. Here, we review recent efforts that aim to address these issues. The approaches are diverse and range from the detailed analysis of interactions between genetic changes and environmental changes in a model transcription factor, to whole-genome investigations of epistatic interactions in complex networks, and exploit ideas from synthetic biology, experimental evolution, and mathematical modeling of cellular networks. These first studies revealed that environmental changes can drastically alter the interaction between two mutations, such that evolutionary paths can switch between being accessible to being inaccessible. At the scale of networks, certain epistatic effects are beginning to be understood mechanistically. The results pave the way to elucidating the evolution of regulatory networks based on a functional understanding of genetic and environmental interactions.

Epistasis within a regulatory protein

Transcriptional regulation is one of the simplest regulatory mechanisms within cells and, therefore, is a good starting

point to explore the interplay between genetic and environmental changes. A recent study [31] zoomed in on one of the best-understood model systems for transcriptional regulation, the *E. coli lac*-repressor (Figure 2A). The authors had previously used experimental evolution to produce *inverse LacI* variants [32]. In contrast to the wild type repressor $LacI_{WT}$, these $LacI_{Inv}$ mutants repressed the *lac* genes in the presence of the ligand isopropyl- β -D-thiogalactopyranoside (IPTG), rather than in its absence. The genetic basis of the inverse response could be traced to three amino acid substitutions within the protein. Fixating these mutations involved a variable selection that alternated between favoring expression and repression of the downstream genes.

This scenario contains the basic ingredients for the adaptive evolution of regulatory responses: a succession of genetic and environmental changes in time. An elementary question that then arises is how these changes relate to each other. If these two types of change do not interact (meaning that their effects on phenotype or fitness are independent), then the specific pattern of environmental changes is immaterial to the genetic obstacles to evolution. However, if they do interact, obstacles that exist in one environment could be lifted in another (Figure 1). Hence, insight into the environment \times genotype interdependencies as well as the precise patterns of environmental change may be critical to understand the evolutionary adaptation of regulatory systems. Note that, in general, organisms may well fail to show adaptive evolution of regulatory responses to multiple environments and, for instance, rather evolve the same phenotypic change across all environments. To explore these issues, all single and double mutants were constructed for three *inverse LacI* variants that had been isolated, and their *lac* operon expression was assayed with and without IPTG.

The analysis showed a drastic effect of the environment on the genetic interactions between pairs of mutations. For half of the pairs, an environmental change turned magnitude epistasis into sign epistasis. Take, for instance, the mutations T258A, which is positioned at the dimerization

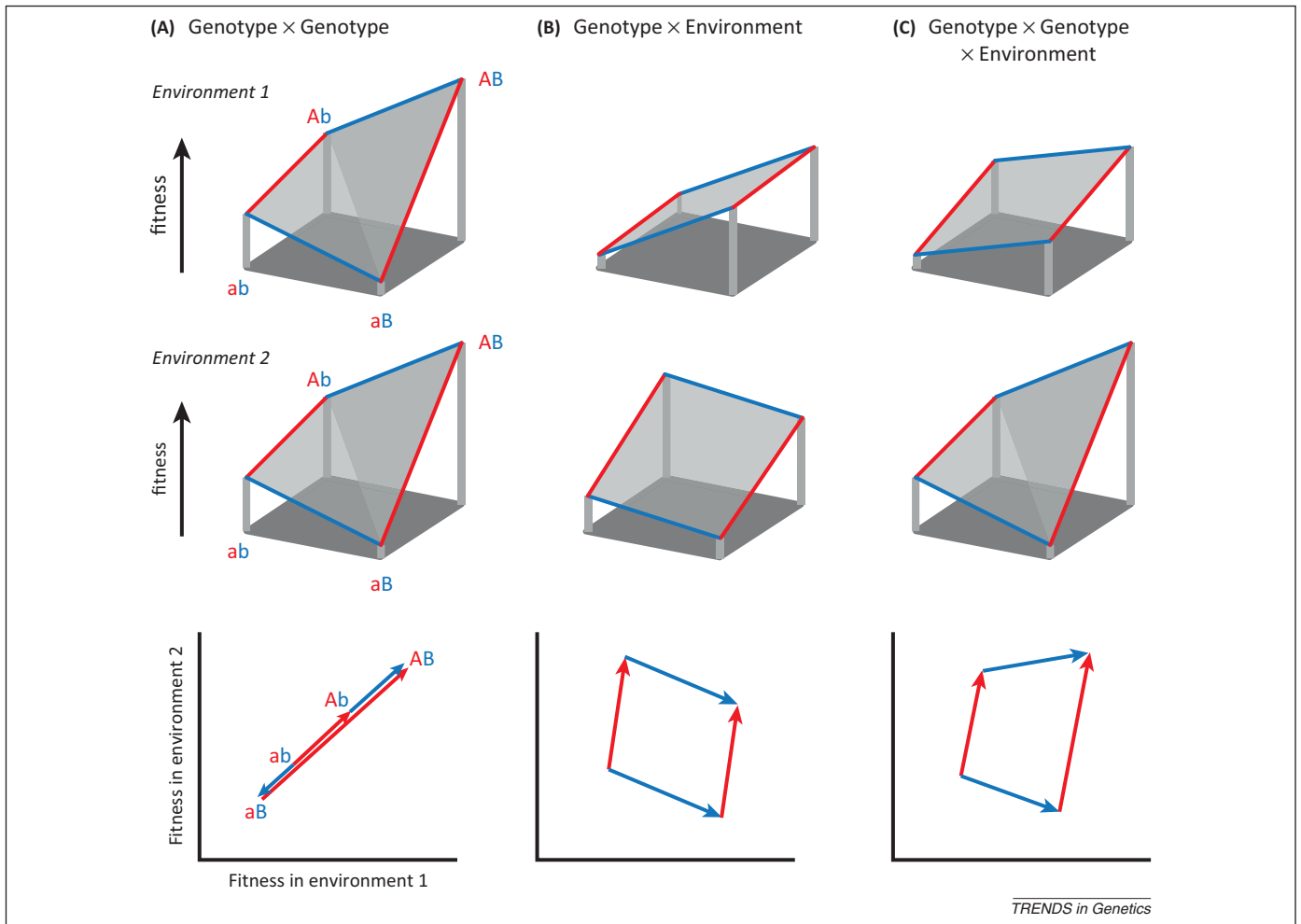


Figure 1. Interdependencies between genotypes (G) and environment (E). The first two rows indicate phenotype or fitness for the elementary genotypes *aa*, *aB*, *Ab*, and *AB* in two environments, where capital denotes a genetic change. The bottom row indicates the same situations, but expressed as vectors in the two-environment fitness space. **(A)** $G \times G$ interactions: the effect of a mutation (*b* to *B*) changes sign when switching to a different genetic background, but remains invariant under environmental changes. Mutation *B* is detrimental when applied to *ab*, but beneficial when applied to *Ab*. **(B)** $G \times E$ interactions: the effect of mutations is identical for different genetic backgrounds, but depends on the environment. In the upper two panels, slopes of opposing lines are identical but are altered by the environment, resulting in a parallelogram in the bottom vector plot. **(C)** $G \times G \times E$ interactions: the effect of a mutation depends on the genetic background as well as the environment. As a result, the type of epistasis is different in both environments. This example shows a change from magnitude epistasis in environment 1 to sign epistasis in environment 2.

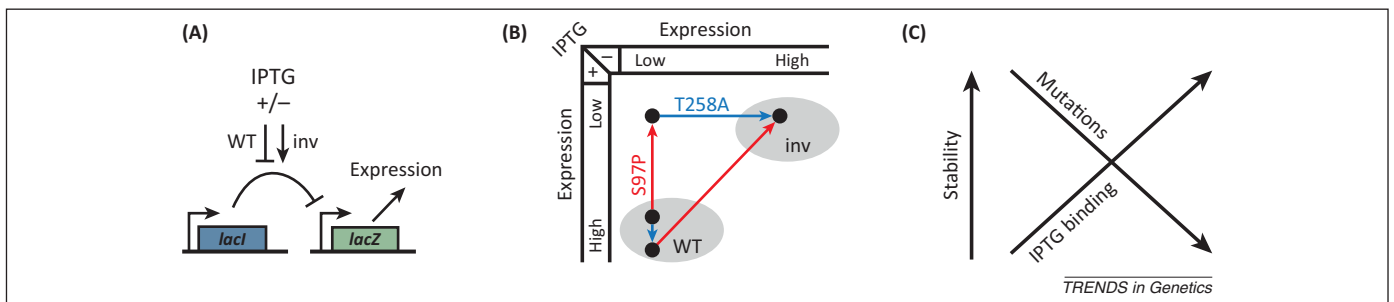


Figure 2. Epistasis and environmental change in the LacI transcriptional regulator. **(A)** Schematic illustration of the synthetic operon and its regulation system. Isopropyl- β -D-thiogalactopyranoside (IPTG) is an inducer for the wild type (WT) LacI repressor and a co-repressor for the evolved inverse (*inv*) LacI repressor. **(B)** LacI mutations displaying genotype × genotype × environment ($G \times G \times E$) interactions (Figure 1, panel C). Prior to S97P, T258A increases LacZ expression, but only in the presence of IPTG. After S97P, the same mutation again increases expression, but now only in the absence of IPTG. **(C)** Proposed mechanistic explanation of the inversion and the higher-order $G \times G \times E$ interactions. First, S97P disrupts the allosteric transition and locks LacI in the DNA-bound state. All mutations decrease LacI stability, leading to increased LacZ expression without IPTG. Stability can be restored by IPTG binding, resulting in repression of LacZ expression.

interface, and S97P, which is positioned in a region where bonds are formed and broken during the LacI conformational change that occurs upon ligand binding. When added to a third mutation, these genetic changes produced an inversion effect (Figure 2). Without IPTG, T258A and S97P were individually neutral, but jointly produced the

required large expression increase. With IPTG, the situation was almost the opposite, because T258A individually increased expression, but was neutral when occurring after S97P. S97P here only decreased expression regardless of T258A. The particular positions of these mutations do not readily provide clues to understand this interaction, or how

they produce inversion. More generally, the complexity of such higher-order interactions between the environment and genotypes is difficult to grasp intuitively.

However, the relative simplicity of the system did open the door to a mechanistic explanation of the observed inversion and the involved higher-order interactions (Figure 2C). First, the mutations were found to decrease overall structural stability, which may have little effect on function until a critical destabilization is reached [33–35]. Thus, both mutations individually had almost no effect on DNA binding or LacZ expression in the absence of IPTG, but jointly conferred enough destabilization to break down DNA binding and increase expression. Second, S97P is known to block the allosteric change and keep LacI in the DNA-binding conformation [36–38]. This explains why S97P is neutral without IPTG but confers an expression decrease with IPTG. Finally, the binding of IPTG can restore stability in the destabilized LacI and, hence, result in DNA binding and repression [31]. This analysis indicates that a combination of simple molecular effects can give rise to a complex pattern of interactions between mutations and environmental change.

The higher-order interactions can directly impact selection. For instance, the mutations T258A and S97P are beneficial only when occurring simultaneously in the absence of IPTG, which dramatically lowers their chance of fixation. By contrast, positive selection is opened up when switching to IPTG, because S97P is then individually beneficial. However, T258A is not beneficial with IPTG, and so another environmental switch is required to access inversion by positive selection. A note of caution is that selection acts on fitness and not on expression. At the same time, a monotonic relation between expression and fitness would not affect this qualitative analysis. Thus, overall, the environment not only defines selective pressures, but can also modulate underlying genetic constraints.

Variable environments and epistasis can also have non-intuitive effects on the evolutionary dynamics in the absence of regulation. One example is the reversibility of evolution, which has been studied using the antibiotic resistance protein TEM β -lactamase [28]. Two environments containing different antibiotics, taxime or piperacillin with clavulanic acid, favored two different genotypes of TEM β -lactamase. Some evolutionary intermediates displayed trade-offs, meaning that they were only well adapted to one of the two environments. The data showed that such trade-offs can enable reverse evolution, because deleterious mutations in one environment became beneficial in the second environment. Nevertheless, it was observed that reversibility of evolution was limited. Even though all transitions containing only one mutation were found to be reversible, the same did not hold true in general: reversibility as well as the accessibility of evolutionary transitions decreased when the length of the mutational path increased.

Natural evolution in changing environments

Even though coping with changing environments is considered a central cellular function, laboratory experiments of evolution by competition have only just begun to address environmental variability. Long-term experimental evolution studies, such as those of Lenski and coworkers [39], are

based on many replicates of serial transfers of a culture into fresh medium and have yielded a wealth of data [3]. More recently, the same group performed such serial transfer experiments while alternating between different carbon sources [40]. Replicate populations showed larger variations in fitness than those evolved in constant environments, indicating divergent evolution. This finding suggests that evolution in variable environments is more complex than in constant environments, and may allow for a larger variety of evolutionary trajectories to be sampled. Another serial transfer study in variable environments [41] revealed that, whereas the evolving *lac* regulatory system adapted quickly to constant environments, alternating environments produced only either overall expression changes or constitutive expression. This result suggests another added complication, namely that variable environments and the associated increased number of evolutionary objectives lead to more severe constraints.

An alternative solution to the challenge of survival in variable environments is the evolution of stochastic rather than regulated phenotype changes. Examples include bacterial persistence [42] and phase variation [43]. A serial transfer experiment [44] provided some insight into the evolution of phenotype switching. *Pseudomonas fluorescens* bacteria were subjected to a variable selection regime continually favoring the emergence of new phenotypes by excluding the phenotype dominating the previous selection round. Although one may expect such a selection regime to give rise to continuous innovation, two of the 12 replicates of the experiment instead yielded 'bet-hedging' genotypes that displayed stochastic switching between two distinct phenotypes. This finding is reminiscent of the phenotype switching driven by highly mutable loci that is observed in various pathogens, often speculated to be a bet-hedging response to evade the immune system [43].

Fluctuating selection is relevant in ecosystems subject to variations such as diurnal, tidal, or seasonal cycles, intermittent nutrient availability, or rare catastrophic events. Ecosystems may also be subject to gradual change, for instance as a result of resource depletion, pollution, or climate change. The rate of environmental change has been shown to affect survival of *E. coli* populations in an experimental evolution assay [29]. Replicate populations underwent serial dilutions into media with a gradually increasing concentration of an antibiotic. Under such conditions where mutations are rare and selection is strong, every single mutation must be beneficial to be retained. Thus, mutational trajectories are selectively accessible only if they comprise a series of single mutations, each of which increases fitness. Similar to previous work [45–48], fewer population extinctions were found for lower rates of environmental change. This finding is not surprising because demographics alone ensure that populations that spend more time under a lower concentration of antibiotic have more opportunity to produce beneficial mutations. However, by genetically tracing out the mutational trajectories of some of the successful lineages and measuring fitness for all intermediates in all environments, the authors showed that their experiment was dominated by a different mechanism: some of the mutational trajectories taken by the populations that successfully evolved through the slowly changing environment

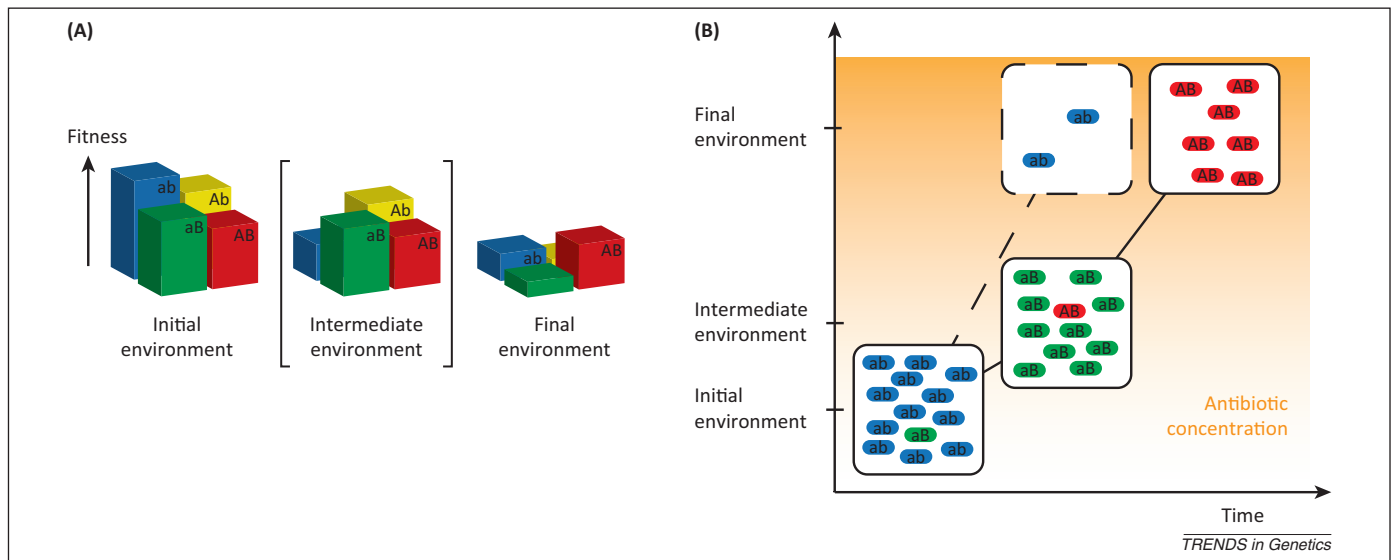


Figure 3. Historical contingency upon an intermediate environment. **(A)** Consider mutational trajectories from the ancestor genotype *ab* to *AB* via the intermediates *aB* or *Ab*. *ab* is fitter than its neighbors in the initial and final environment, but *AB* is fitter than either in the final environment. If no intermediate environment is provided in which *aB* or *Ab* will be positively selected over the initial genotype *ab*, then these two trajectories to *AB* are not selectively accessible. They are historically contingent upon the intermediate environment. **(B)** The demographics of an example population moved from the initial to the final environment either with (solid lines) or without time spent in the intermediate environment (broken lines). In the latter case (broken lines), the most successful genotype for the final environment cannot evolve.

were ‘historically contingent’ upon intermediate environments. In essence, this means that the path taken is not selectively accessible without this intermediate environment (Figure 3). It was also shown that such historical contingencies not only require the gene \times gene ($G \times G$) effect of sign epistasis, but also a dependence of the sign of the fitness effect of a mutation on the environment (i.e., $G \times G \times E$).

In this study, the identification of relevant mutations was possible because the resistance-conferring mutations were limited to a single gene. The advent of next generation-sequencing methods will help to identify functionally relevant mutations that are distributed among different genes that function jointly within regulatory networks and, thus, enable similar approaches in more complex systems.

Constraints in cellular networks

We have so far mostly discussed genetic and environmental interactions at the level of individual genes. However, most cellular responses to environmental signals (from metabolism to chemotaxis to differentiation) rely on elaborate networks, which raises the issue of interdependencies between different genes. At the most elementary level, for instance, regulatory molecules that physically contact each other by a lock-and-key mechanism have been shown to give rise to intergenic epistasis [21,49]. At a more systems level, epistatic interactions have been studied in metabolic networks, for instance. Here, we review these and other efforts to understand epistasis and evolutionary constraints in cellular networks, both from the modeling and experimental evolution perspectives.

One of the modeling approaches simulated the effects of single and double knockouts in yeast metabolism, using a flux balance analysis model [50]. Such models maximize the total flux among all possible solutions under known constraints of the system, the network topology being accounted for by stoichiometric relations. Here, interactions were classified into three categories: negative

epistasis, no epistasis, or positive epistasis, corresponding to the deleterious effect of combined mutations being stronger, equal to, or weaker than the additive expectation, respectively. It was observed that the most relevant scale to classify epistasis was at the level of metabolic modules, defined as sets of genes that contribute to the same metabolic pathway, such as glycolysis or ATP synthesis. Even though interactions within modules contributed significantly to epistasis, most epistatic interactions were measured to occur between modules, suggesting the importance of functional relations between them. Moreover, these interactions were observed mainly between genes without known physical interactions. The central finding of this study was that, with few exceptions, genes from the same metabolic module had the same type of epistatic interaction with genes from another module. Conversely, the authors clustered genes by maximizing epistasis similarity between clusters and again found consistent functional sets, showing the independence of this finding from previous gene annotations.

Some of these key predictions were later verified by experimental screens of double mutants in yeast [51,52]. The authors developed a protocol to infer fitness from colony size and measured the effect of several million double knockouts, including metabolic genes. They found that proteins participating in the same complex had mostly the same type of epistatic interactions with proteins from another complex. It was also confirmed that a large amount of epistasis occurred between genes that were not known to engage in direct physical interactions.

Evolution experiments have also produced evidence for the importance of functional interactions between genes in evolutionary trajectories. In one such study, the authors performed more than 100 parallel repeats of the adaptation of bacteria to elevated temperature [53], and they sequenced genomes of individuals randomly picked from each independent line of evolution. They found that only 2–3% of point mutations were shared between two or more

lines, whereas the evolutionary patterns were reproducible at higher levels of organization in the cell. Indeed, mutations were strongly biased toward 12 functional modules, such as the RNA polymerase complex, the wall formation complex, the stress regulation genes, as well as specific metabolic pathways.

The dominance of these few functional modules in shaping the evolutionary trajectories was confirmed when looking at mutation distributions in more details. In fact, a majority of these modules contained a single mutation per line, which was significantly less than the random expectation. Moreover, when considering a given module across different lines, the observed single mutations affecting it were diverse, emphasizing the importance of functional modules as evolutionary units, rather than specific mutations within them. The authors interpreted the occurrence of a single mutation per module as negative epistasis, when the combined beneficial effect of two mutations was almost equal to the effect of only one mutation. In the simplest case, for instance, adaptation by inactivating a gene can be achieved by several mutations, but once one such damaging mutation is fixed, additional mutations in the same gene would be neutral and, hence, not be fixed. Note that such negative epistasis also appeared in situations where genes retained their function, pointing to additional mechanisms that remain to be explained. In any case, further adaptation could occur only by changes in other functional modules.

Two genes deviated from the general result and typically sustained more than two mutations per independent line. These were the transcription termination factor *rho* and the *rpoBC* operon, encoding subunits of the RNA polymerase. As suggested by the authors, the broad range of functions that these two genes affect explains the existence of several compensatory mutations. More importantly, mutations in these two genes were systematically accompanied by mutations in other modules, the set of modules associated with either *rho* or *rpoBC* having strikingly small overlap. This suggested the existence of a structured fitness landscape, with mutations occurring primarily in either *rho* or *rpoBC* constraining further evolution to separated evolutionary paths. The authors measured that these two paths led to the same level of fitness, further suggesting the existence of two local optima and sign or reciprocal sign epistasis between these two exclusive sets of mutations. These findings are not isolated and other evolutionary studies have recently shown negative epistasis [54,55] and sign or reciprocal sign epistasis [22,56,57] between genes within metabolic pathways or at a global level of organization in the cell.

Note that the existence of a relation between network structure and epistasis is not a priori obvious, because even simple network topologies can give rise to a variety of epistasis patterns depending on details of the network components [49,58]. Such relations may in particular be averaged out when one tries to find correlations between epistasis and statistical quantifications of network complexity or local connectivity [58,59], which may disregard some specific structures at intermediate and large scales of the network. By clustering genes, such specific structures were captured, eventually leading to the classification of epistasis patterns [50].

Concluding remarks

Environmental change and the genetic makeup of organisms are considered key to evolution. Indeed, biologists since Darwin have been intrigued by the emergence of novel regulatory functions in populations challenged by the conflicting demands of variable environments [60]. However, phenotypes that have been studied historically have been too complex to link environment and genetics at the molecular level. Here, we have provided some insight into how these obstacles are now being overcome by new approaches that combine modern genetics and synthetic biology with an appreciation of simple model systems. The first results indicate not only that biological systems display pervasive interdependencies, but that these interdependencies can also critically affect their evolution. Changes in the environment do not merely affect the magnitude of genetic epistasis, but can completely reshuffle genetic interactions. As a result, evolutionary paths that are blocked in one environment can be opened up in another and, vice versa, paths that are accessible in one environment can become blocked in another. Thus, environmental change can drive evolutionary transitions by altering genetic interactions.

The pervasiveness of E×G interactions raises many questions. For instance, patterns of environmental variation are ecologically diverse, ranging from gradual changes to stochastic fluctuations and spatial structured variations. These patterns can produce diverse evolutionary responses, consisting of gain or loss of regulatory abilities, bet-hedging, and impact on divergence within populations. Many open issues remain at the scale of regulatory networks, for instance on the relation between functional and evolutionary constraints. So far, the focus has been on constant environments, leaving the regime of variable environments to be explored. Theoretical approaches are being developed that use a simplified phenotype-fitness mapping [61] or dynamic simulations of networks [62,63] to assess epistasis. Such conceptual developments could be coupled with targeted network modifications and quantitative phenotypic assays [64]. Together, such efforts can provide a new perspective on the variety of observed regulatory functions and behavior, and allow for a quantitative understanding of evolution in complex environments.

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References

- Dill, K.A. *et al.* (2011) Physical limits of cells and proteomes. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17876–17882
- Wright, S. (1932) The roles of mutation, inbreeding, crossbreeding and selection in evolution. *Proc. Sixth Cong. Genet.* 1, 356–366
- Elena, S.F. and Lenski, R.E. (2003) Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* 4, 457–469
- Weinreich, D.M. *et al.* (2006) Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* 312, 111–114
- Poelwijk, F.J. *et al.* (2007) Empirical fitness landscapes reveal accessible evolutionary paths. *Nature* 445, 383–386

- 6 Weinreich, D.M. *et al.* (2005) Perspective: sign epistasis and genetic constraint on evolutionary trajectories. *Evolution* 59, 1165–1174
- 7 Bridgham, J.T. *et al.* (2006) Evolution of hormone–receptor complexity by molecular exploitation. *Science* 312, 97–101
- 8 Pigliucci, M. (2001) *Phenotypic Plasticity: Beyond Nature and Nurture*, John Hopkins University Press
- 9 Gottesman, S. (1984) Bacterial regulation: global regulatory networks. *Annu. Rev. Genet.* 18, 415–441
- 10 Kern, D. and Zuiderweg, E.R.P. (2003) The role of dynamics in allosteric regulation. *Curr. Opin. Struct. Biol.* 13, 748–757
- 11 Rosenfeld, S. (2011) Mathematical descriptions of biochemical networks: stability, stochasticity, evolution. *Prog. Biophys. Mol. Biol.* 106, 400–409
- 12 Riechmann, J.L. *et al.* (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science* 290, 2105–2110
- 13 Atchley, W.R. and Fitch, W.M. (1997) A natural classification of the basic helix-loop-helix class of transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5172–5176
- 14 Babu, M.M. *et al.* (2004) Structure and evolution of transcriptional regulatory networks. *Curr. Opin. Struct. Biol.* 14, 283–291
- 15 Lusk, R.W. and Eisen, M.B. (2010) Evolutionary mirages: selection on binding site composition creates the illusion of conserved grammars in *Drosophila* enhancers. *PLoS Genet.* 6, e1000829
- 16 Wittkopp, P.J. and Kalay, G. (2012) *Cis*-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. *Nat. Rev. Genet.* 13, 59–69
- 17 Richards, F.M. (1977) Areas, volumes, packing, and protein structure. *Annu. Rev. Biophys. Bioeng.* 6, 151–176
- 18 Kirby, D.A. *et al.* (1995) Maintenance of pre-mRNA secondary structure by epistatic selection. *Proc. Natl. Acad. Sci. U.S.A.* 92, 9047–9051
- 19 DePristo, M.A. *et al.* (2005) Missense meanderings in sequence space: a biophysical view of protein evolution. *Nat. Rev. Genet.* 6, 678–687
- 20 Phillips, P.C. (1996) Waiting for a compensatory mutation: phase zero of the shifting-balance process. *Genet. Res.* 67, 271–283
- 21 Poelwijk, F.J. *et al.* (2011) Reciprocal sign epistasis is a necessary condition for multi-peaked fitness landscapes. *J. Theor. Biol.* 272, 141–144
- 22 Kvitck, D.J. and Sherlock, G. (2011) Reciprocal sign epistasis between frequently experimentally evolved adaptive mutations causes a rugged fitness landscape. *PLoS Genet.* 7, e1002056
- 23 de Visser, J.A.G.M. *et al.* (2009) Exploring the effect of sex on empirical fitness landscapes. *Am. Nat.* 174 (Suppl. 1), S15–S30
- 24 Remold, S.K. and Lenski, R.E. (2004) Pervasive joint influence of epistasis and plasticity on mutational effects in *Escherichia coli*. *Nat. Genet.* 36, 423–426
- 25 Kubinak, J.L. *et al.* (2012) Experimental viral evolution to specific host MHC genotypes reveals fitness and virulence trade-offs in alternative MHC types. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3422–3427
- 26 Bataillon, T. *et al.* (2011) Cost of adaptation and fitness effects of beneficial mutations in *Pseudomonas fluorescens*. *Genetics* 189, 939–949
- 27 Remold, S. (2012) Understanding specialism when the Jack of all trades can be the master of all. *Proc. Biol. Sci.* 279, 4861–4869
- 28 Tan, L. *et al.* (2011) Hidden randomness between fitness landscapes limits reverse evolution. *Phys. Rev. Lett.* 106, 198102
- 29 Lindsey, H.A. *et al.* (2013) Evolutionary rescue from extinction is contingent on a lower rate of environmental change. *Nature* 494, 463–467
- 30 Hall, A.R. *et al.* (2011) The fitness cost of rifampicin resistance in *Pseudomonas aeruginosa* depends on demand for RNA polymerase. *Genetics* 187, 817–822
- 31 de Vos, M.G.J. *et al.* (2013) Environmental dependence of genetic constraint. *PLoS Genet.* 9, e1003580
- 32 Poelwijk, F.J. *et al.* (2011) Tradeoffs and optimality in the evolution of gene regulation. *Cell* 146, 462–470
- 33 Chen, P. and Shakhnovich, E.I. (2009) Lethal mutagenesis in viruses and bacteria. *Genetics* 183, 639–650
- 34 Tokuriki, N. *et al.* (2008) How protein stability and new functions trade off. *PLoS Comput. Biol.* 4, e1000002
- 35 Wylie, C.S. and Shakhnovich, E.I. (2011) A biophysical protein folding model accounts for most mutational fitness effects in viruses. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9916–9921
- 36 Flynn, T.C. *et al.* (2003) Allosteric transition pathways in the lactose repressor protein core domains: asymmetric motions in a homodimer. *Protein Sci.* 12, 2523–2541
- 37 Zhan, H. *et al.* (2010) Positions 94–98 of the lactose repressor N-subdomain monomer–monomer interface are critical for allosteric communication. *Biochemistry* 49, 8636–8645
- 38 Lewis, M. *et al.* (1996) Crystal structure of the lactose operon repressor and its complexes with DNA and inducer. *Science* 271, 1247–1254
- 39 Lenski, R.E. and Travisano, M. (1994) Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc. Natl. Acad. Sci. U.S.A.* 91, 6808–6814
- 40 Cooper, T.F. and Lenski, R.E. (2010) Experimental evolution with *E. coli* in diverse resource environments. I. Fluctuating environments promote divergence of replicate populations. *BMC Evol. Biol.* 10, 10
- 41 Poelwijk, F.J. *et al.* (2011) Optimality and evolution of transcriptionally regulated gene expression. *BMC Syst. Biol.* 5, 128
- 42 Balaban, N.Q. (2011) Persistence: mechanisms for triggering and enhancing phenotypic variability. *Curr. Opin. Genet. Dev.* 21, 768–775
- 43 van der Woude, M.W. and Baumberg, A.J. (2004) Phase and antigenic variation in bacteria. *Clin. Microbiol. Rev.* 17, 581–611
- 44 Beaumont, H.J. *et al.* (2009) Experimental evolution of bet hedging. *Nature* 462, 90–93
- 45 Perron, G.G. *et al.* (2008) The rate of environmental change drives adaptation to an antibiotic sink. *J. Evol. Biol.* 21, 1724–1731
- 46 Bell, G. and Gonzalez, A. (2009) Evolutionary rescue can prevent extinction following environmental change. *Ecol. Lett.* 12, 942–948
- 47 Collins, S. and de Meaux, J. (2009) Adaptation to different rates of environmental change in *Chlamydomonas*. *Evolution* 63, 2952–2965
- 48 Bell, G. and Gonzalez, A. (2011) Adaptation and evolutionary rescue in metapopulations experiencing environmental deterioration. *Science* 332, 1327–1330
- 49 Lehner, B. (2011) Molecular mechanisms of epistasis within and between genes. *Trends Genet.* 27, 323–331
- 50 Segrè, D. *et al.* (2005) Modular epistasis in yeast metabolism. *Nat. Genet.* 37, 77–83
- 51 Baryshnikova, A. *et al.* (2010) Quantitative analysis of fitness and genetic interactions in yeast on a genome scale. *Nat. Methods* 7, 1017–1024
- 52 Costanzo, M. *et al.* (2010) The genetic landscape of a cell. *Science* 327, 425–431
- 53 Tenaillon, O. *et al.* (2012) The molecular diversity of adaptive convergence. *Science* 335, 457–461
- 54 Khan, A.I. *et al.* (2011) Negative epistasis between beneficial mutations in an evolving bacterial population. *Science* 332, 1193–1196
- 55 Chou, H-H. *et al.* (2011) Diminishing returns epistasis among beneficial mutations decelerates adaptation. *Science* 332, 1190–1192
- 56 Woods, R.J. *et al.* (2011) Second-order selection for evolvability in a large *Escherichia coli* population. *Science* 331, 1433–1436
- 57 Damkjaer, S. *et al.* (2013) Evolutionary remodeling of global regulatory networks during long-term bacterial adaptation to human hosts. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7766–7771
- 58 Gjuvsland, A.B. *et al.* (2007) Statistical epistasis is a generic feature of gene regulatory networks. *Genetics* 175, 411–420
- 59 Macià, J. *et al.* (2012) The causes of epistasis in genetic networks. *Evolution* 66, 586–596
- 60 DeWitt, T.J. and Scheiner, S.M. (2004) *Phenotypic Plasticity: Functional and Conceptual Approaches*, Oxford University Press
- 61 Chiu, H-C. *et al.* (2012) Epistasis from functional dependence of fitness on underlying traits. *Proc. Biol. Sci.* 279, 4156–4164
- 62 Pumar, A. and Shraiman, B. (2011) Epistasis in a model of molecular signal transduction. *PLoS Comput. Biol.* 7, e1001134
- 63 Cotterell, J. and Sharpe, J. (2013) Mechanistic explanations for restricted evolutionary paths that emerge from gene regulatory networks. *PLoS ONE* 8, e61178
- 64 Carter, G.W. *et al.* (2007) Prediction of phenotype and gene expression for combinations of mutations. *Mol. Syst. Biol.* 3, 96