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Empirical fitness landscapes reveal accessible evolutionary paths

Frank J. Poelwijk[1]*, Daniel J. Kiviet[1]*, Daniel M. Weinreich[2]† & Sander J. Tans[1]

When attempting to understand evolution, we traditionally rely on analysing evolutionary outcomes, despite the fact that unseen intermediates determine its course. A handful of recent studies has begun to explore these intermediate evolutionary forms, which can be reconstructed in the laboratory. With this first view on empirical evolutionary landscapes, we can now finally start asking why particular evolutionary paths are taken.

Evolutionary intermediates represented a central preoccupation for Darwin in his case for the theory of evolution. He remarked, for example: '…why, if species have descended from other species by insensibly fine gradations, do we not everywhere see innumerable transitional forms?'[1]. Although Darwin developed a convincing rationale for their absence, he did realize that the lack of intermediates as proof leaves room for criticism. He noted, for instance: 'If it could be demonstrated that any complex organ existed which could not possibly have been formed by numerous, successive, slight modifications, my theory would absolutely break down.'[1]. Indeed, in their opposition to evolution, the proponents of 'intelligent design' have seized on our current ignorance of intermediates.

Building on earlier ideas[2–4], an approach has recently been developed to explore the step-by-step evolution of molecular functions. The central innovation is that all molecular intermediates along multiple putative pathways are explicitly reconstructed. Together with a phenotypic characterization of each intermediate, one can determine whether paths towards a certain novel function are accessible by natural selection. Although others have reconstructed and characterized phylogenetically ancestral forms of proteins[4–7], here the focus is on fitness landscapes[8] in which multiple mutational trajectories can be compared. Fitness landscapes have been widely studied on a theoretical level (see refs 9–13 for example), but one can now obtain a glimpse of actual biological landscapes. This view finally allows us to ask which particular evolutionary paths are taken and why. In particular, to what extent do biomolecular properties constrain evolution? Does it matter in which order mutations occur? Are fitness landscapes rugged, with many local optima acting as evolutionary dead-ends, or are they smooth? Is neutral genetic drift essential for a new trait to emerge?

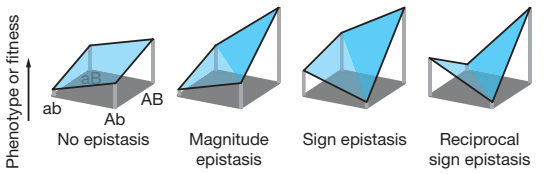
When examining the molecular underpinnings of the evolution of new traits, we distinguish two elementary cases. First, we discuss a single mutable component such as an enzyme. Second, we look at molecular interactions involving two or more mutable components, which is typical for regulatory evolution. The specific features of this broad range of molecular systems will be discussed using the notions of epistasis and fitness landscapes, which we will explain and relate to each other (Box 1 and Fig. 1).

The tentative picture emerging from the new results is one that emphasizes the possibilities of continuous optimization by positive selection. Although evolution was clearly constrained, as illustrated by many inaccessible evolutionary paths, the studies also revealed alternative accessible routes: a succession of viable intermediates exhibiting incremental performance increases. Although these findings do not address whether natural evolution proceeds in the presence or absence of selection, they do show that neutral genetic drift is not essential in the cases studied. We note that the presented approach starts with naturally occurring sequences, which are

Box 1 | Epistasis and the accessibility of mutational paths

Epistasis means that the phenotypic consequences of a mutation depend on the genetic background (genetic sequence) in which it occurs. In the Box figure we distinguish four cases that illustrate paths composed of two mutations, from the initial sequence 'ab' towards the optimum at 'AB'. When there is no epistasis, mutation 'a' to 'A' yields the same fitness effect for different genetic backgrounds ('b' or'B'), while for magnitude epistasis the fitness effect differs in magnitude, but not in sign. For sign epistasis, the sign of the fitness effect changes. Finally, such a change in sign of the fitness effect can occur for both mutations, which we here term reciprocal sign epistasis.

These distinctions are crucial in the context of selection. Mutations exhibiting magnitude epistasis or no epistasis are always favoured (or disfavoured), regardless of the genetic background in which they appear. In contrast, mutations exhibiting sign epistasis may be rejected by natural selection, even if they are eventually required to increase fitness. In other words, some paths to the optimum contain fitness decreases, while other paths are monotonically increasing. When all paths between two sequences contain fitness decreases, there are two or more distinct peaks. The presence of multiple peaks indicates reciprocal sign epistasis, and may cause severe frustration of evolution (Fig. 1b). Indeed, reciprocal sign epistasis is a necessary condition for multiple peaks, although it does not guarantee it: the two optima in the diagram may be connected by a fitness-increasing path involving mutations in a third site.



[1]FOM Institute AMOLF, Kruislaan 407, 1098 SJ, Amsterdam, The Netherlands. [2]Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138, USA. †Present address: Department of Ecology and Evolutionary Biology, and Center for Computational Molecular Biology, Brown University, Providence, Rhode Island 02192, USA.
*These authors contributed equally to this work.

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themselves the product of evolution, and may therefore yield a biased sample of trajectories. Whether the conclusions are general or not, and whether they break down when the evolved feature becomes more complex, can only be determined through future studies.

Enzyme evolution

When a well-adapted organism is challenged by a new environment, an existing gene may perform suboptimally. One of the most basic questions one may then ask is: when mutating step-by-step from the suboptimal to an optimal allele, are all possible trajectories selectively accessible? This question depends critically on the stepwise changes in performance, or in fitness, which are governed by unknown physical and chemical properties at the molecular level. When all mutations along all paths yield a fitness improvement, evolution can rapidly proceed in a straightforward incremental darwinian fashion. In this case, the fitness landscape can be portrayed by a single smooth peak (Fig. 1a).

Whether this picture is realistic was investigated for the adaptation of bacterial β-lactamase to the novel antibiotic cefotaxime[14]. The central step was to reconstruct and measure all likely intermediates, allowing a systematic study of all possible trajectories. The intermediate sequences can be easily identified, because the (five) mutations that control the cefotaxime resistance phenotype are known, resulting in $2^5 = 32$ possible mutants. The order in which the mutations are fixed can of course be different, giving rise to $5! = 120$ possible direct trajectories between the start and end sequences.

The trajectory analysis showed that the fitness landscape is not as simple as depicted in Fig. 1a. A majority of the pathways towards maximum cefotaxime resistance actually shows a dip in fitness (see yellow path in Fig. 1b), or contain selectively neutral steps (as in Fig. 1c), resulting in much smaller chances of being followed by natural selection[12,15]. For 18 paths however, each step appeared to confer a resistance increase, making these trajectories accessible to darwinian selection. The part of the fitness landscape mapped out in this manner therefore does appear to have a single peak, but one that contains depressions and plateaus on its slopes. We stress that such three-dimensional analogies, while useful for conveying basic characteristics, do not rigorously represent the many direct trajectories existing between two alleles. Also note that there may be additional paths that contain detours, involving other mutations that are eventually reverted[16] (Fig. 1d).

Interestingly, some mutations yielded either a resistance increase or decrease, depending on the preceding mutations. This phenomenon, called sign epistasis[13] (see Box), is both a necessary and sufficient condition for the fitness landscape to contain inaccessible paths to an optimum[13]. Some cases of sign epistasis could be understood in terms of competing molecular mechanisms. For instance, a first mutation in the wild-type enzyme increased the resistance by enhancing the catalytic rate, even though it also lowered the thermodynamic stability. This loss of stability was repaired by a second mutation, thereby further increasing the resistance. In contrast, when this 'stabilizing' mutation occurred first in the wild-type enzyme, the resistance was reduced. Such back and forth balancing between structural and functional benefits might well be a more general evolutionary mechanism[17,18].

In a second study[19], the connection between fitness landscape and underlying molecular properties has been explored for the evolution of isopropylmalate dehydrogenase (IMDH, Fig. 2a), an enzyme that is involved in the biosynthesis of leucine. As in the previous study, a set of mutational intermediates between different functions were characterized. Here the mutations changed the cofactor binding affinity of IMDH. *In vitro* measurements of enzyme activity did not show epistasis: each mutation gave a fixed catalytic improvement, which was independent of the order in which they occurred. Thus, the 'enzyme activity' landscape is single-peaked.

The story becomes more complete with the following elements. First, the study also considered evolutionary paths from the suboptimal cofactor NADP to the normal cofactor NAD[20]. Second, selection does not act directly on enzyme activity, but rather on the fitness of an organism. As fitness is typically nonlinear in enzyme activity, epistasis is introduced. Therefore, the IMDH mutants were also evaluated *in vivo*, providing a direct measurement of the fitness effect of a mutation. The resulting fitness landscape was shown to contain a depression or valley, rendering the trajectories that pass through it

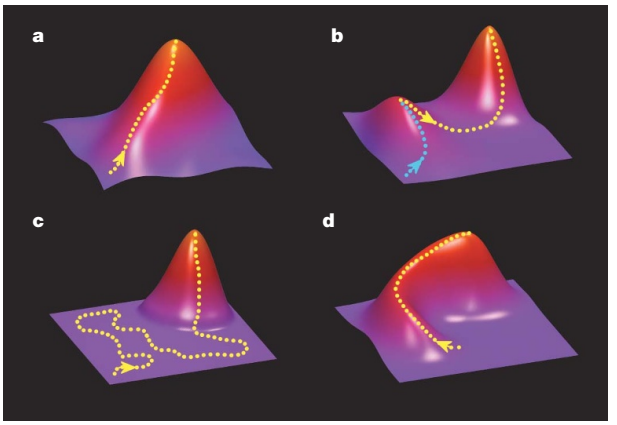

Figure 1 | Schematic representations of fitness landscape features. Fitness is shown as a function of sequence: the dotted lines are mutational paths to higher fitness. **a**, Single smooth peak. All direct paths to the top are increasing in fitness. **b**, Rugged landscape with multiple peaks. The yellow path has a fitness decrease that drastically lowers its evolutionary probability. Along the blue path selection leads in the wrong direction to an evolutionary trap[16]. **c**, Neutral landscape. When neutral mutations are essential, evolutionary probabilities are low[12,15]. **d**, Detour landscape. The occurrence of paths where mutations are reverted[16] shows that sequence analysis may fail to show mutations that are essential to the evolutionary history.

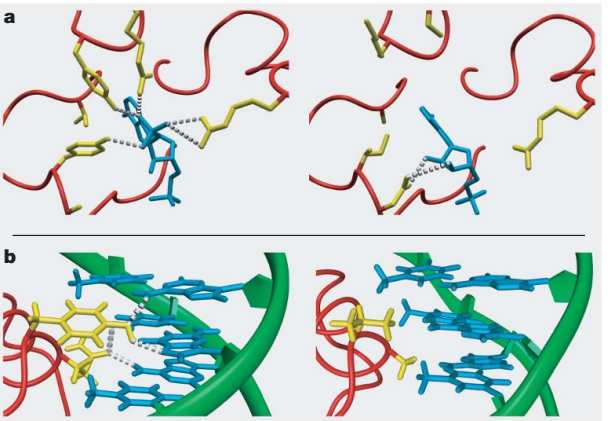

Figure 2 | Molecular structures in different evolutionary forms. Main chains are shown in red, key residues in yellow, the DNA backbone in green, key DNA bases or cofactor in blue, and hydrogen bonds as dashed lines. **a**, The left panel shows wild-type *E. coli* isocitrate dehydrogenase[34] (IDH), which is structurally similar to IMDH, with NADP as cofactor. The right panel shows an engineered IDH form with NAD as cofactor[35]. **b**, The left panel shows a wild-type *E. coli lac* repressor and operator[36]. The right panel shows a *lac* repressor and operator variant, with mutations mimicking the *gal* system[37]. Binding is tight and specific (despite the absence of hydrogen bonds): these variants bind wild-type partners poorly. Figures prepared with MOLMOL[38].

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selectively inaccessible. There is an intuitive rationale for a valley here: when the recognition of NADP is reduced, the fitness first decreases, before it rises again when NAD recognition is built up. Interestingly however, some trajectories also exist that avoid the valley by simultaneously increasing NAD, and decreasing NADP recognition. In the end, the genotype–fitness landscape has a single peak, but one that includes a depression on its slope.

Evolution of molecular interactions

The evolutionary puzzle becomes more complex at a higher level of cellular organization. In the web of regulatory interactions between ligands, proteins and DNA, the components are strongly interdependent, which might suggest that their evolution is severely constrained. The evolution of molecular recognition has recently been explored by two studies, which also used experimentally reconstructed intermediates. The first examined hormone detection by steroid receptors in the basal vertebrates (Fig. 3a)[21]. The second[16] looked at the adaptation of repressor–operator binding, in a large evolutionary landscape based on published mutation data for the *Escherichia coli lac* system[22] (Figs 2b and 3b). For both studies, the molecular interactions may be thought of as a key fitting a lock. The unifying question is: can a new lock and matching key be formed taking just one mutational step at a time? The adaptation of these components presents a dilemma: if the lock is modified first, the intermediate is not viable because the old key does not fit, and vice versa.

From the evolution of the interactions in the two systems (Fig. 3), some interesting parallels are apparent. Both studies start with a duplication event yielding two locks and keys, and then ask how specific interactions can be obtained during mutational divergence. Specificity is clearly vital: two partners must recognize each other, but not recognizing other components is just as important. A major evolutionary challenge is therefore to decrease unwanted interactions, while maintaining desired interactions. Without specific hormone recognition, cortisol regulation of vertebrate metabolism, inflammation and immunity would be perturbed by varying levels of aldosterone, which controls electrolyte homeostasis. Similarly, specific recognition in the *lac* family of repressors allows *E. coli* to consume a wide array of sugars, without the burden of producing many unused metabolic enzymes.

Surprisingly, these studies again show that new interactions can evolve in a step-by-step darwinian fashion, despite the mismatching intermediates problem sketched above. In the hormone receptor case, this predicament is overcome by a molecular version of a master key: a putative ancestral ligand, 11-deoxycorticosterone, was found to activate all receptors (ancestral and present-day), allowing the mutational intermediates to remain functional even while the receptors diverged (Fig. 3a). The capability to synthesize aldosterone evolved later, finally providing a specific hormone that is recognized by just one of the two receptors. An existing receptor was thus recruited into a new role, as a binding partner to aldosterone, in a process that was termed 'molecular exploitation'. Sign epistasis was again observed: an initial mutation drastically lowered the response to all substrates, but after another mutation, the same mutation improved cortisol response while decreasing the aldosterone response. Thus, just as in the β-lactamase and IMDH cases, at least one selectively accessible evolutionary pathway existed.

In the evolution of the *lac* system, a similar mechanism using a 'master' repressor or operator was not observed. This is illustrated by the transient loss in affinity during the adaptation from one tight repressor–operator pair (IM–TG) to another (IK–AC); see Fig. 3b. Between some alleles, all connecting paths transiently reduced the affinity, indicating the presence of multiple peaks in the affinity landscape, which contrasts with the single-peaked landscapes of β-lactamase and IMDH. Multiple peaks indicate a severe kind of sign epistasis, which we here term reciprocal sign epistasis (see Box 1). Reciprocal sign epistasis can be intuitively understood for molecular interactions: mutating one binding partner will probably only benefit a new interaction if the other binding partner is mutated first, and vice versa. Interestingly, this means that although sign epistasis does introduce landscape ruggedness and thus perturbs the adaptive search, it can also be valuable because it enables multiple independent lock–key combinations.

If the *lac* repressor–operator affinity landscape is rugged and multi-peaked, how can new recognition evolve in a step-by-step manner? The answer lies in the fact that selection does not act on a single interaction. Instead, multiple interactions in a network determine the regulation, and ultimately organismal fitness. In the *lac* case, deteriorations in one interaction were offset by improvements in another. For example, initial mutations in one repressor duplicate were bad for binding to its designated operator, but good for relieving an undesired cross-interaction (Fig. 3b). These results substantiate the suggestion that network robustness[23] may promote evolvability[24,25]. The observed compensations yielded a smoothened fitness landscape, making the new interactions selectively accessible. In fact,

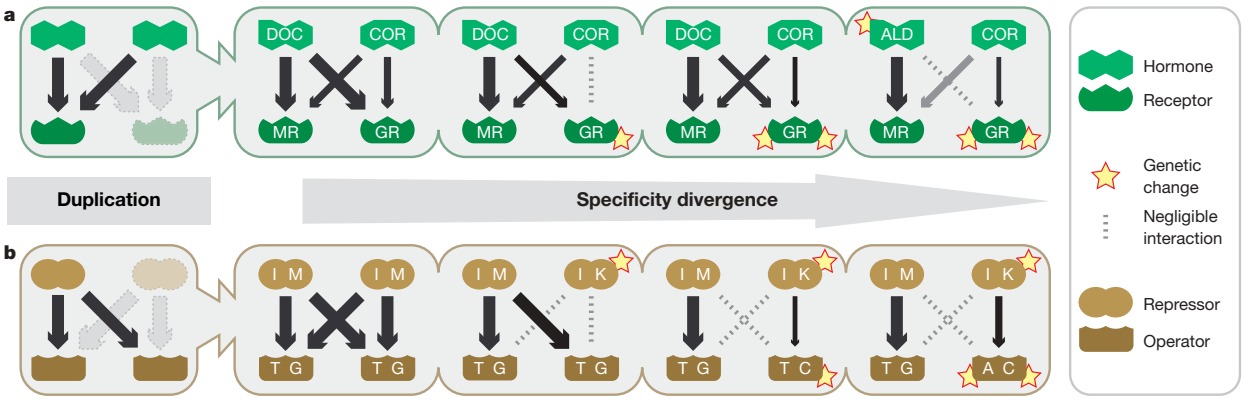

Figure 3 | Evolution of molecular interactions based on reconstructed intermediates. Arrow thickness denotes measured interaction strengths. DOC, 11-deoxycorticosterone; COR, cortisol; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; ALD, aldosterone. **a**, Pathway towards independent steroid receptors after duplication, via intermediate receptors that remained sensitive to their ligands[21]. A changed mutation order produced a non-sensitive intermediate, making that path inaccessible. The grey arrow indicates that cortisol is absent in MR-expressing tissues. **b**, Pathway towards independent repressor–operator pairs following duplication, taking single-mutation steps without decreases in network performance. Many paths were compared in a landscape based on over 1,000 *lac* mutants[22], covering all substitutions on all key base pairs. For simplicity, the repressor dimer and two operator half-sites are not drawn.

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because compensation within biochemical networks is ubiquitously observed[26], we expect that evolution by network compensation constitutes a general mode of regulatory adaptation, molecular interdependence notwithstanding.

Outlook

The experimental reconstruction of evolutionary intermediates and putative pathways has provided an exciting first look at molecular adaptive landscapes. Although numerous paths appear to be selectively inaccessible, accessible pathways are generally also available. Importantly, various alternative types of fitness landscapes were not observed. The landscapes could have been so rugged and multipeaked, that accessible paths to optima would not exist, thus requiring, for instance, two or more simultaneous mutations, larger genetic modifications through recombination, or periods of relaxed selection. We have put forward various mechanisms that can reduce landscape ruggedness and improve evolvability. These include the interplay between protein function and stability[14,19], the exploitation of existing molecules into new roles[21], and compensation within biochemical networks[16].

That only a few paths are favoured also implies that evolution might be more reproducible than is commonly perceived, or even be predictable. It is important to note that evolutionary speed and predictability are not determined only by molecular constraints, but also by population dynamics. Population dynamics still presents many open questions, in particular in the context of regulatory evolution and varying environments. The situation in which environmental fluctuations are fast relative to selection timescales has been explored in the repressor divergence study[16]. Recent theoretical considerations[27,28] may provide promising approaches to address these questions more generally.

The molecular systems interrogated so far represent only a start, but one with great potential to spark further exploration. The analysis of intermediates is generally applicable, which makes finding new candidate systems not difficult. Mutational paths could also be revealed using the directed evolution methodology[29], in which randomly mutated pools are screened. A related approach is the experimental evolution[30] of cells in chemostats[31] or by serial dilution[32,33]. The advantage of these methods is that more extensive and unbiased evolutionary changes can be explored, although they do not directly reveal why trajectories are chosen. Together, these developments may change the character of molecular evolution research from one that is primarily sequence-based to one that explicitly incorporates structure, function and fitness.

1. Darwin, C. *On the Origin of Species by Means of Natural Selection* Ch VI (Murray, London, 1859).
2. Pauling, L. & Zuckerkandl, E. Chemical paleogenetics; Molecular "restoration studies" of extinct forms of life. *Acta Chem. Scand. A* **17,** S9–S16 (1963).
3. Maynard Smith, J. Natural selection and the concept of a protein space. *Nature* **225,** 563–564 (1970).
4. Malcolm, B. A. *et al.* Ancestral lysozymes reconstructed, neutrality tested, and thermostability linked to hydrocarbon packing. *Nature* **345,** 86–89 (1990).
5. Stackhouse, J., Presnell, S. R., McGeehan, G. M., Nambiar, K. P. & Benner, S. A. The ribonuclease from an extinct bovid ruminant. *FEBS Lett.* **262,** 104–106 (1990).
6. Ugalde, J. A., Chang, B. S. W. & Matz, M. V. Evolution of coral pigments recreated. *Science* **305,** 1433 (2004).
7. Thornton, J. W. Resurrecting ancient genes: Experimental analysis of extinct molecules. *Nature Rev. Genet.* **5,** 366–375 (2004).
8. Wright, S. The roles of mutation, inbreeding, crossbreeding and selection in evolution. *Proc 6th Int. Cong. Genet.* **1,** 356–366 (1932).
9. Gillespie, J. H. *The Causes of Molecular Evolution* (Oxford Univ. Press, Oxford, 1991).
10. Kauffman, S. A. *The Origins of Order: Self-organization and Selection in Evolution* (Oxford Univ. Press, Oxford, 1993).
11. Gavrilets, S. *Fitness Landscapes and the Origin of Species* (Princeton Univ. Press, Princeton, 2004).
12. van Nimwegen, E. & Crutchfield, J. P. Metastable evolutionary dynamics: crossing fitness barriers or escaping via neutral paths? *Bull. Math. Biol.* **62,** 799–848 (2000).
13. Weinreich, D. M., Watson, R. A. & Chao, L. Sign epistasis and genetic constraint on evolutionary trajectories. *Evol. Int. J. Org. Evol.* **59,** 1165–1174 (2005).
14. Weinreich, D. M., Delaney, N. F., DePristo, M. A. & Hartl, D. L. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* **312,** 111–114 (2006).
15. Kimura, M. On the probability of fixation of mutant genes in a population. *Genetics* **47,** 713–719 (1962).
16. Poelwijk, F. J., Kiviet, D. J. & Tans, S. J. Evolutionary potential of a duplicated repressor-operator pair: simulating pathways using mutation data. *PLoS Comput. Biol.* **2,** e58 (2006).
17. DePristo, M. A., Weinreich, D. M. & Hartl, D. L. Missense meanderings in sequence space: a biophysical view of protein evolution. *Nature Rev. Genet.* **6,** 678–687 (2005).
18. Bloom, J. D., Labthavikul, S. T., Otey, C. R. & Arnold, F. H. Protein stability promotes evolvability. *Proc. Natl Acad. Sci. USA* **103,** 5869–5874 (2006).
19. Lunzer, M., Miller, S. P., Felsheim, R. & Dean, A. M. The biochemical architecture of an ancient adaptive landscape. *Science* **310,** 499–501 (2005).
20. Zhu, G., Golding, G. B. & Dean, A. M. The selective cause of an ancient adaptation. *Science* **307,** 1279–1282 (2005).
21. Bridgham, J. T., Carroll, S. M. & Thornton, J. W. Evolution of hormone-receptor complexity by molecular exploitation. *Science* **312,** 97–101 (2006).
22. Lehming, N., Sartorius, J., Kisters-Woike, B., von Wilcken-Bergmann, B. & Müller-Hill, B. Mutant lac repressors with new specificities hint at rules for protein-DNA recognition. *EMBO J.* **9,** 615–621 (1990).
23. Barkai, N. & Leibler, S. Robustness in simple biochemical networks. *Nature* **387,** 913–917 (1997).
24. Kirschner, M. & Gerhart, J. Evolvability. *Proc. Natl Acad. Sci. USA* **95,** 8420–8427 (1998).
25. Kitano, H. Biological robustness. *Nature Rev. Genet.* **5,** 826–837 (2004).
26. Stelling, J., Sauer, U., Szallasi, Z., Doyle, F. J. III & Doyle, J. Robustness of cellular functions. *Cell* **118,** 675–685 (2004).
27. Thattai, M. & van Oudenaarden, A. Stochastic gene expression in fluctuating environments. *Genetics* **167,** 523–530 (2004).
28. Kussell, E. & Leibler, S. Phenotypic diversity, population growth, and information in fluctuating environments. *Science* **309,** 2075–2078 (2005).
29. Arnold, F. H., Wintrode, P. C., Miyazaki, K. & Gershenson, A. How enzymes adapt: lessons from directed evolution. *Trends Biochem. Sci.* **26,** 100–106 (2001).
30. Elena, S. F. & Lenski, R. E. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Rev. Genet.* **4,** 457–469 (2003).
31. Couñago, R., Chen, S. & Shamoo, Y. *In vivo* molecular evolution reveals biophysical origins of organismal fitness. *Mol. Cell* **22,** 441–449 (2006).
32. Lenski, R. E. & Travisano, M. Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc. Natl Acad. Sci. USA* **91,** 6808–6814 (1994).
33. Dekel, E. & Alon, U. Optimality and evolutionary tuning of the expression level of a protein. *Nature* **436,** 588–592 (2005).
34. Hurley, J. H., Dean, A. M., Koshland, D. E. Jr & Stroud, R. M. Catalytic mechanism of NADP(+)-dependent isocitrate dehydrogenase: implications from the structures of magnesium-isocitrate and NADP+ complexes. *Biochemistry* **30,** 8671–8678 (1991).
35. Hurley, J. H., Chen, R. & Dean, A. M. Determinants of cofactor specificity in isocitrate dehydrogenase: structure of an engineered NADP+ → NAD+ specificity-reversal mutant. *Biochemistry* **35,** 5670–5678 (1996).
36. Kalodimos, C. G. *et al.* Plasticity in protein-DNA recognition: lac repressor interacts with its natural operator 01 through alternative conformations of its DNA-binding domain. *EMBO J.* **21,** 2866–2876 (2002).
37. Kopke Salinas, R. *et al.* Altered specificity in DNA binding by the lac repressor: a mutant lac headpiece that mimics the gal repressor. *ChemBioChem* **6,** 1628–1637 (2005).
38. Koradi, R., Billeter, M. & Wüthrich, K. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14,** 51–55 (1996).

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