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Evolvability

A Unifying Concept in Evolutionary Biology?

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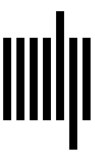

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11 Mutational Robustness and Evolvability

Andreas Wagner

Organisms are to some extent robust to DNA mutations: Their phenotypes do not change in the face of some DNA mutations that affect the gene(s) encoding these phenotypes. Robustness can facilitate evolvability—the ability of a biological system to produce phenotype variation that is both heritable and adaptive. Here I first introduce some concepts that are necessary to understand why robustness can entail evolvability. I then discuss empirical evidence that speaks to the relationship between robustness and evolvability, focusing on systems where the molecular foundations of both robustness and evolvability can be studied in detail. Finally, I discuss empirical evidence that robustness can itself evolve, and that evolvability mediated by robustness can itself be subject to adaptive evolution.

11.1 Introduction

Organisms are to some extent robust to DNA mutations. That is, their phenotypes do not change in the face of some DNA mutations that affect the gene(s) encoding these phenotypes, or the regulatory DNA driving the expression of these genes (Wagner 2005; Masel and Siegal 2009; Fares 2015). This robustness can vary among organisms, among phenotypes, and among the genotypes encoding any one phenotype (Giver et al. 1998; Lynch and Conery 2000; Salazar et al. 2003; Bloom et al. 2006; Fasan et al. 2008; Jiménez et al. 2013; Keane et al. 2014; Payne and Wagner 2014, 2019; Najafabadi et al. 2017; Starr et al. 2017; Payne et al. 2018; Vaishnav et al. 2021). Such mutational or genetic robustness is closely linked to evolvability—the ability to bring forth phenotypic variation that is both heritable and adaptive (Wagner 2005, 2008; Draghi et al. 2010; Mayer and Hansen 2017; Payne and Wagner 2019). At first sight, this relationship might seem straightforward: High robustness implies that a given number of mutations generate little phenotypic variation—adaptive or otherwise—and because natural selection requires phenotypic variation, high robustness should imply low evolvability. The argument is simple, but it is also misleading. In fact, high robustness often entails high evolvability. In this chapter, I will first explain why, and then discuss pertinent empirical evidence.

This is not an exhaustive review of the relevant literature, which could easily fill an entire book (Wagner 2005). For example, I do not discuss the role of recombination in the evolution of robustness, nor do I say much about the role of robustness to environmental change. I also omit scenarios where robustness is not adaptive, because selection favors genotypic and phenotypic diversity. Examples include the antibody diversity that helps the

adaptive immune system combat pathogens. They also include the antigenic diversity that numerous pathogens create through targeted recombination or mutation processes, which help them evade host immune responses (Deitsch et al. 2009). Furthermore, I do not discuss the burgeoning theoretical literature on robustness. Instead, I provide a few key ideas that link mutational robustness and evolvability, and I discuss the empirical evidence supporting this link. More specifically, I first introduce some concepts that are necessary to understand why robustness can entail evolvability. I then discuss pertinent empirical evidence, focusing on systems where the molecular foundations of both robustness and evolvability can be studied in great detail. Finally, I discuss what we know about the evolution of robustness and evolvability.

11.2 Concepts

To understand the relationship between robustness and evolvability, it is essential to know that the same phenotype is usually encoded by many different genotypes in a *genotype space*. Such a space is typically defined as the set of all DNA or amino acid sequences of a given length. If these sequences are short, genotype space comprises a modest number of genotypes. Consider, for example, the regions of regulatory DNA known as transcription factor binding sites. Such sites are typically shorter than 16 base pairs (bps; Stewart and Plotkin 2012) and thus exist in a genotype space of fewer than $4^{16} \approx 4 \times 10^9$ molecules. When a transcriptional activator binds to such a site, its binding can help turn on a nearby gene's transcription in proportion to its binding affinity. The phenotype of such a site is its ability to bind the activator, which depends on its DNA sequence (genotype). Any one transcription factor can bind dozens to hundreds of such DNA sequences with similar affinity, and thus activate a nearby gene to a similar extent (Badis et al. 2009; Weirauch et al. 2014).

For more complex biomolecules, both genotype space and the number of genotypes encoding the same phenotype can be much larger (Reidhaar-Olson and Sauer 1990; Schuster et al. 1994; Reidys et al. 1997; Keefe and Szostak 2001). To give an example, consider RNA molecules of length $L = 30$ nucleotides, which constitute a genotype space of $4^{30} \approx 10^{18}$ RNA sequences. Their minimum free energy secondary structure phenotypes—the planar folds they can form through internal base pairing—are biologically important, because they are essential for the biological functions of many RNA molecules (Baudin et al. 1993; Powell et al. 1995). Most such RNA phenotypes are encoded by multiple RNA genotypes, and the number of genotypes encoding the same phenotype varies by several orders of magnitude among phenotypes (Wagner 2008).

Analogous observations hold for proteins. For example, it has been estimated experimentally that $\approx 10^{93}$ amino acid sequences of length 80 amino acids are able to bind ATP (Keefe and Szostak 2001). Likewise, more than 10^{56} amino acid sequences of length $L = 93$ encode the λ repressor, a transcriptional regulator of bacteriophage λ (Reidhaar-Olson and Sauer 1990). These numbers are unimaginably large, but they still constitute a vanishing fraction of genotype space. For example, in the genotype space of 20^{93} proteins with $L = 93$ amino acids, the 10^{56} λ repressors constitute a fraction $\approx 10^{-63}$ of genotype space.

Robustness does not just require that multiple genotypes encode the same phenotype. It also requires that any one genotype G has multiple *1-mutant neighbors* with this phenotype. A 1-mutant neighbor of G is a genotype that can be created from it by a single DNA

mutation, such as a single nucleotide change. I will refer to the collection of all 1-mutant neighbors of a genotype G as G's (1-mutant) *neighborhood.* Such a neighborhood comprises $3L$ genotypes for DNA or RNA molecules of length L, because each of the 4 possible nucleotides can mutate into 3 other nucleotides, and $19L$ genotypes for proteins of length L amino acids, if each of the 20 proteinaceous amino acid can mutate into 19 others.

The smaller a genotype's fraction of 1-mutant neighbors with the same phenotype is, the smaller will be the robustness of this genotype. Figure 11.1a illustrates this idea in a highly simplified schematic of a hypothetical genotype G whose phenotype is indicated by the black circle in the center. This genotype has 8 1-mutant neighbors (connected to it by thick black lines), all of which are assumed to encode a different phenotype (shapes at the tip of each line) than G itself. Thus, this genotype is minimally robust to mutations. Figure 11.1b shows another hypothetical genotype G with 8 neighbors, but only 3 of these neighbors have a different phenotype. The other 5, connected to G by gray lines, encode the same phenotype (not shown) as G itself. The genotype in figure 11.1b is more robust than that in figure 11.1a. Under the assumption that among all neighboring phenotypes, some (possibly small) fraction of them is adaptive, high robustness of a genotype implies low evolvability. I will refer to this notion of robustness and evolvability as *genotypic* robustness and evolvability, because they are properties of a specific genotype encoding a phenotype (Wagner 2008). I emphasize that figure 11.1 is an abstraction to illustrate general ideas with simplifications chosen for the purpose of explanation. For example, many phenotypes are continuous rather than categorical quantities, and one genotype may encode more than one phenotype.

Although the negative association between robustness and evolvability appears inevitable from a theoretical perspective, it is reassuring that it also has empirical support. Pertinent evidence comes from an experiment that measured the ability of 20 million yeast regulatory regions (genotypes) of $L = 80$ bp to activate the expression of a yeast gene in a massively parallel assay. The phenotype of any one such sequence is the expression level of the regulated gene. The experimenters then used a deep learning neural network to predict this phenotype for those sequences whose regulatory activity they had not measured. Subsequently, they synthesized and tested thousands of further regulatory sequences and showed that the network's predictions are in excellent agreement with experimental data. With this tool in hand, the authors defined a regulatory sequence's (genotypic) evolvability as a mutation's tendency to change the expression level of the regulated gene. Not surprisingly, mutationally robust sequences were less evolvable (Vaishnav et al. 2021).

To see the limitations of these genotype-centered concepts, consider again some genotype G encoding a phenotype, such as a protein's ability to catalyze a chemical reaction. Consider a single 1-mutant neighbor of G with the same phenotype, such as genotype G_2 in figure 11.1c. This 1-mutant neighbor may itself have multiple neighbors that preserve this phenotype (one of which is shown as G_2 in figure 11.1c), which in turn may themselves have multiple neighbors with the same phenotype, and so on. In other words, the genotypes encoding the same phenotype may form a network in genotype space.

For such a network to be large and extend far through genotype space, it is sufficient that the genotypes encoding any one phenotype must have a modest nonzero fraction of neighbors that encode the same phenotype (Reidys et al. 1997; Wagner 2011). Such networks have first been described in computational models of protein and RNA folding

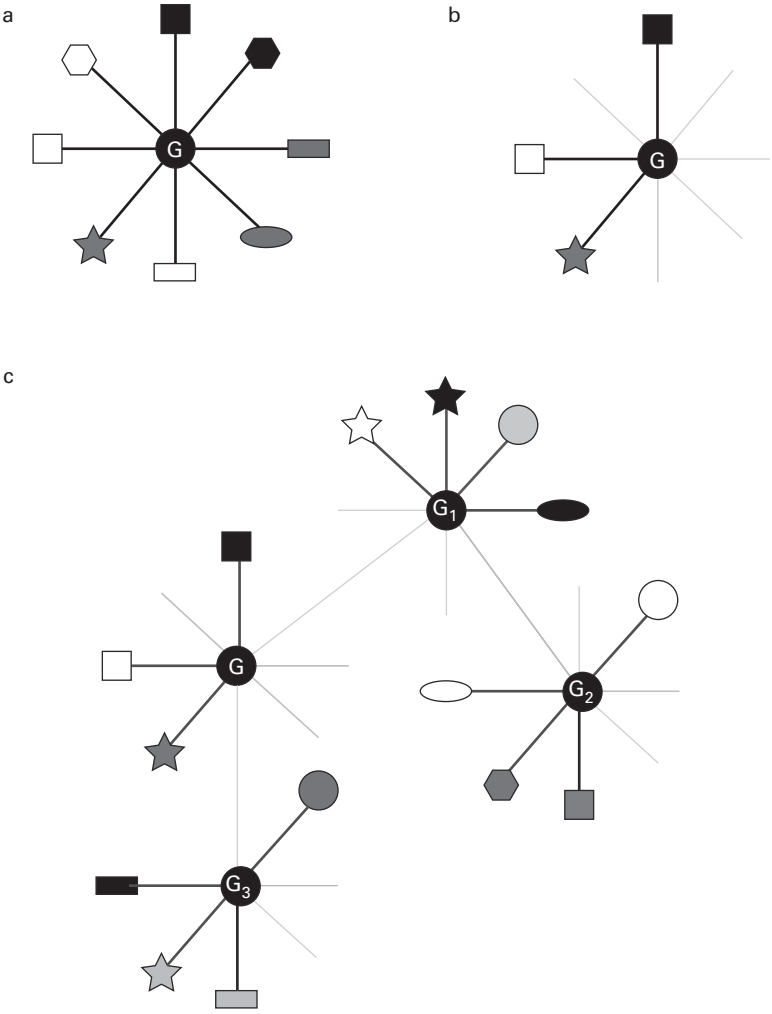

Figure 11.1
Robustness, genotype networks, and evolvability illustrated with a highly simplified hypothetical example. The figure shows genotypes as nodes in a graph, where neighboring genotypes are connected by straight lines. Different phenotypes are indicated by different shapes and their shading. a) Hypothetical minimally robust genotype G, whose 8 1-mutant neighbors all have a different phenotype. b) As in panel a, but only 3 neighbors have the same phenotype, whereas the remaining 5 neighbors (connected to G by gray lines) have the same phenotype (not shown) as G itself. c) As in panel b, but now the phenotypes in the neighborhood of 2 1-mutant neighbors of G (G_1 and G_3), as well as of 1 2-mutant neighbor (G_2) are also shown. Neighbors with the same phenotype are again connected by gray lines. Even though G is to some extent robust, and thus only 3 novel phenotypes are accessible in its immediate 1-neighborhood, 14 novel phenotypes are accessible from it through $G_1 - G_3$, because these networks form a phenotype-preserving genotype network. This simple schematic neglects the high-dimensional nature of genotype space, the continuous nature of many phenotypes, as well as the fact that many genotypes encode multiple phenotypes, but the key principles hold for more complex scenarios as well (Wagner 2014).

(Lipman and Wilbur 1991; Schuster et al. 1994). However, they exist on all levels of biological organization, not just for proteins and RNA molecules, but also for regulatory circuits and their gene expression phenotypes (Ciliberti et al. 2007; Schaerli et al. 2014), as well as for the chemical reaction networks encoded by metabolic genes and their ability to metabolize specific nutrients (Rodrigues and Wagner 2009).

A well-studied example among proteins is oxygen-binding globins. These ancient proteins share a common ancestor that existed many hundreds of million years ago, and they exist in both animals and plants. They have preserved their protein structure and their biochemical, oxygen-binding phenotype. At the same time, phylogenetic analysis shows that they have dramatically diverged in genotype through single amino acid changes, such that 2 globins may share less than 5% amino acid identity along their coding sequence (Goodman et al. 1988; Hardison 1996). Proteins with highly diverged genotypes and conserved phenotypes are the rule rather than the exception among biological macromolecules (Thornton et al. 1999; Rost 2002; Bastolla et al. 2003).

A population that evolves under mutation and selection acting to preserve an adaptively important phenotype explores genotype space along the kind of network illustrated in figure 11.1c. Based on computational models, such networks have first been called neutral networks (Schuster et al. 1994), suggesting that their exploration involves only neutral mutations. However, this need not be the case. For example, even though a mutation may preserve a globin's oxygen-binding ability, the mutation may increase or decrease this ability, and thus not be neutral with respect to fitness. As long as the mutation is not highly deleterious, however, it may not be eliminated from an evolving population, and it may provide a stepping-stone toward further mutation (Ohta 1992; Eyre-Walker et al. 2002; Kern and Kondrashov 2004; Kulathinal et al. 2004; Weinreich and Chao 2005; Sawyer et al. 2007). Because strict neutrality is not required for the exploration of a network of genotypes, I prefer to call such networks more generically *genotype networks*.

Genotype networks—a consequence of robustness—can facilitate evolvability. They allow an evolving population to explore a broad region of genotype space through mutations that preserve its phenotype, which may be important if the phenotype is vital for survival. During this process, the population's members also explore the mutational neighborhoods of multiple genotypes on a genotype network. In the hypothetical example shown in figure 11.1c, a total of 14 different novel phenotypes (shapes) are accessible to G via G_1, G_2, and G_3, even though genotype G is quite robust (i.e., the 1-neighborhood of G contains, just like in figure 11.1b only 3 novel phenotypes). Many more novel phenotypes may be accessible through further neighbors of these genotypes.

If different neighborhoods contain different novel phenotypes, the chances of encountering an adaptive novel phenotype can be much greater than through the exploration of just a single neighborhood. To illustrate the dramatic increase in the number of novel phenotypes that can become accessible through a genotype network, consider a guide RNA from *Trypanosoma brucei* with $L = 40$ nucleotides and its minimum free energy secondary structure phenotype (accession number L25590 of the functional RNA database (https://dbarchive.biosciencedbc.jp/en/frnadb). The 1-neighborhood of this genotype G comprises $3L = 120$ genotypes and could thus encode at most 120 different phenotypes. However, computational predictions of RNA secondary structures show that only 40 of these neighbors encode a novel phenotype (Wagner 2012). In other words, G is to some extent robust

to mutations, and this robustness reduces the number of novel phenotypes that are accessible in its immediate (1-mutant) neighborhood. However, the 1-mutant neighborhoods of G's 1-mutant neighbors encode many more novel phenotypes, 746 to be precise (Wagner 2012). In other words, just 2 mutations away from G 746, new phenotypes become accessible. Furthermore, the 1-mutant neighborhoods of all 2-mutant neighbors of G contain an even greater number of 1,174 distinct new phenotypes (Wagner 2012). Thus in just a few mutational steps away from a focal genotype, the total number of accessible novel phenotypes escalates rapidly. The total number of genotypes forming this guide RNA's secondary structure can be computed, and it is greater than 10^{17} (Jörg et al. 2008). It is not currently possible to compute the total number of novel phenotypes in the neighborhoods of all these genotypes, but this number is likely to be astronomically large as well.

These considerations show that it is shortsighted to just consider the robustness and evolvability of *genotypes*. Instead, the robustness and evolvability of *phenotypes* may be more useful. One can define the *robustness of a phenotype* as the average fraction of a genotype's neighbors with this phenotype, where the average is taken over all genotypes encoding this phenotype. Likewise, one can define the average *evolvability of a phenotype* as the total number of novel phenotypes that can be found in the neighborhoods of all genotypes encoding this phenotype. Some fraction of these novel phenotypes will be adaptive. Because more robust phenotypes have larger genotype networks, an evolving population with such a phenotype thus can access more genotype neighborhoods, which contain more novel phenotypes than the accessible neighborhoods of a less robust phenotype. In other words, phenotypic robustness can entail phenotypic evolvability.

This has first been shown computationally for RNA secondary structure phenotypes (Wagner 2008), but relevant empirical evidence exists for other systems (Ferrada and Wagner 2008; Payne and Wagner 2014). For example, consider the DNA binding sites of eukaryotic transcription factors (TF), where genotype networks have been studied for 104 mouse and 89 yeast TFs (Payne and Wagner 2014). A typical TF binds multiple DNA sequences with high affinity, and this number varies among factors, from dozens to hundreds of sites (Badis et al. 2009; Weirauch et al. 2014). For 99% of the examined factors, the majority of a factor's binding sites formed a single connected genotype network. The average robustness of these sites varied broadly among factors, ranging between 7% and 48% of a site's 1-mutant neighbors that were bound by the same factor (Payne and Wagner 2014). Larger genotype networks are formed by factors with more robust binding sites. The neighborhood of a TF's genotype network—the collection of all binding sites that are 1 nucleotide change away from at least one of the network's genotypes—harbors binding sites for multiple other TFs. If one uses the number of such novel binding sites as a measure of evolvability, high robustness entails high evolvability, that is, a larger repertoire of new binding sites that are only 1 nucleotide change away from a given genotype network (Payne and Wagner 2014).

I emphasize that the concepts introduced so far abstract from a complex reality and are subject to several caveats (De Visser et al. 2003; Meyers et al. 2005; Manrubia and Cuesta 2015; Mayer and Hansen 2017). For example, they statically enumerate genotypes with specific phenotypes and ignore the dynamics of evolving populations. Most genotype networks are much larger than any one population evolving on it, so that such a population

will only be able to explore a tiny region of such a network, even on time scales of millions of years. Thus, viewing robustness and evolvability as averages over all genotypes in a network may arguably be less important than examining them in a region that an evolving population can explore. This is especially important if there is substantial variation in robustness and evolvability among different regions of a genotype network. Such variation indeed exists. For example, whereas on average, 37% of DNA sequences that bind the mouse TF Foxa2 are robust to single nucleotide changes, this percentage varies enormously among individual binding sites and ranges from 3% to 72% (Payne and Wagner 2014). Where such local variation is extreme, the relationship between robustness and evolvability may change. For example, it has been proposed that evolving populations may become entrapped in regions of genotype space where any one genotype is so highly robust that most of its neighbors have the same genotype (Manrubia and Cuesta 2015). Such entrapment can lead to low evolvability. This possibility is to date only theoretical, but it illustrates that local or regional properties of a genotype space have the potential to affect the relationship between robustness and evolvability. The starting point and duration of an evolutionary process, together with other parameters, such as the mutation rate and population size, can all potentially influence the relationship between robustness and evolvability.

For these reasons, it is important to study this relationship with empirical evidence derived from evolving populations. Two principal approaches provide such evidence. The first is experimental evolution, where whole organisms or molecules are evolved in the laboratory or in vitro. Such experiments, combined with high-throughput genotyping as well as physiological and biochemical analyses of evolved genotypes and their phenotypes, can examine the evolutionary process in real time and in exquisite molecular detail. One of their limitations is that they are restricted to short evolutionary time scales and to populations that explore only small regions of a genotype space. This time limitation imposes further constraints, such as the necessity to work at high mutation rates or at selection pressures that may be stronger than in the wild.

The second approach comprises comparative and phylogenetic studies that infer past evolutionary processes from extant organisms. It can be aided by the reconstruction of ancestral and extinct genotypes, and by biochemical analyses of these phenotypes (Bridgham et al. 2006; Dean and Thornton 2007; Ortlund et al. 2007; Eick et al. 2012; McKeown et al. 2014; Anderson et al. 2015; Nocedal et al. 2017; Starr et al. 2017, 2018). This approach can overcome the limitations of experimental evolution, but it has its own limitations, which come from its need to infer the past from the present. In the next section, I discuss data from both approaches, which show that robustness can facilitate evolvability.

11.3 Empirical Data Show that Robustness Facilitates Evolvability

One fundamental consequence of robustness is that evolving populations can accumulate *cryptic genetic variation*. This is genetic variation that does not cause phenotypic variation while it accrues but is not phenotypically neutral under all circumstances. It can give rise to novel phenotypic variation when the environment changes, or when further mutations arise (Rutherford and Lindquist 1998; True and Lindquist 2000; Masel and Bergman 2003;

True et al. 2004; Masel 2006; Jarosz and Lindquist 2010). Evolution experiments have been used to ask whether cryptic variation can facilitate adaptive evolution. They can help explain the role of robustness in adaptive evolution (Tokuriki and Tawfik 2009; Hayden et al. 2011; Rigato and Fusco 2016; Zheng et al. 2019). In one pertinent experiment, my colleagues and I used directed evolution to accumulate cryptic variation in a yellow fluorescent protein (YFP). The experiment employed repeated cycles ("generations") of mutation and selection on the yellow fluorescent light emitted by YFP. Specifically, we evolved 4 populations of YFP under strong stabilizing selection on the native yellow fluorescence phenotype, which allowed the population to accumulate cryptic genetic variation with minimal effect on the light-emitting phenotype. After 4 generations of stabilizing selection, we continued for another 4 generations but under strong directional selection for a new color phenotype, namely, green fluorescence. In parallel, we evolved 4 additional populations toward green fluorescence, but these populations had not been given the opportunity to accumulate cryptic genetic variation. We found that populations with cryptic variation evolved green fluorescence more rapidly than those without it (Zheng et al. 2019). In addition, populations with cryptic variation evolved a higher intensity of green fluorescence (Zheng et al. 2019). Moreover, populations with cryptic evolution evolved a greater diversity of green-fluorescing genotypes. In sum, this experiment not only shows that cryptic variation facilitates evolutionary adaptation. It also shows that robustness can help evolving populations find diverse (and superior) solutions to the adaptive problems they face. The reasons are easy to understand from the visual metaphor of figure 11.1c: Robustness implies that evolving populations can diversify in multiple directions from a starting genotype, and each of these directions may lead to different high-fitness genotypes.

Cryptic variation can also facilitate evolvability in other systems, and most notably in whole organisms. In one pertinent experiment, Rigato and Fusco (2016) used chemical mutagenesis to introduce a modest number (<30) mutations into the genome of *E. coli* cells. They then subjected populations of the mutagenized cells for 56 generations to strong stabilizing selection on their ability to grow on glucose. The purpose of this procedure was to eliminate phenotypic variation that may have been caused by mutagenesis and thus to preserve only cryptic variation in the populations. In addition, Rigato and Fusco also exposed populations without prior mutagenesis to strong stabilizing selection for the same number of generations. At the end of this preparatory experiment, both kinds of populations showed the same (low) amount of phenotypic variation in their growth rate on glucose. If any additional genetic variation that the mutagenized populations harbored was cryptic, then its phenotypic effects should be revealed in the right environment or genetic background. This was indeed the case, as the researchers' next experiment showed. In this experiment, the researchers subjected both the mutagenized and nonmutagenized populations to directional selection for different phenotypes, namely, high growth on lactate or glycerol (in separate experiments). The mutagenized populations adapted faster to both glycerol and lactate (Rigato and Fusco 2016). Furthermore, the researchers showed through mutagenesis that growth on glycerol is more robust to mutations than is growth on lactate, and that populations adapted more rapidly to glycerol than to lactate. In other words, the more robust phenotype was also more evolvable (Rigato and Fusco 2016). Note that the experiments discussed here involved large populations and a single change of the selective environment (Rigato and Fusco

2016; Zheng et al. 2019). Cryptic variation may affect adaptive evolution differently in smaller populations and frequently changing environments.

Other experimental studies focused on comparing the evolvability of systems with high and low robustness (Bloom et al. 2006, 2010; McBride et al. 2008; Igler et al. 2018; Zakrevsky et al. 2021). One such experiment created populations of RNA bacteriophage φ6, whose ability to survive and reproduce was either robust or sensitive to mutations (Montville et al. 2005; McBride et al. 2008). The experiment created these populations by serially passaging viral populations through the bacterium *Pseudomonas syringae* under conditions where bacteria were either infected by a single virus or simultaneously by multiple viruses. (Multiple co-infections can help a defective virus reproduce, because its defects can be complemented by other, intact co-infecting virus. During the course of multiple passages through a host, such complementation causes viral populations to become more sensitive to mutations.) The experimenters then evolved both types of populations toward increased survivorship after heat shocks of 45°C. Specifically, they passaged these viruses through bacteria for 50 viral generations and exposed them to a heat shock every 5 generations. The more robust populations adapted more rapidly to the heat shock treatment (McBride et al. 2008).

A completely different kind of experiment revolves around cytochrome P450, a class of enzymes that can catalyze reactions with multiple substrates. In this experiment, Bloom and collaborators engineered a cytochrome P450 enzyme for higher thermodynamic stability by introducing a specific stabilizing mutation into the enzyme (Bloom et al. 2006). This mutation also increases the robustness of this enzyme's activity to mutations. The researchers then asked whether the modified enzyme could more easily evolve the ability to catalyze reactions with new substrates. To find out, they introduced random mutations into the enzyme variants with high and low robustness, at an average incidence of 4.5 nucleotide changes per P450-coding gene. They then monitored the ability of both variants to catalyze reactions with multiple substrates. The more robust variants showed higher activity after mutagenesis on several substrates (Bloom et al. 2006).

All of these experiments rely on the short time scales of laboratory evolution. Phylogenetic analyses can help elucidate the relationship between robustness and evolvability on much longer time scales (Ferrada and Wagner 2008; Najafabadi et al. 2017; Nocedal et al. 2017; Starr et al. 2017). One such analysis focused on the 3-dimensional folds (tertiary structure) of ancient and well-studied enzymes (Ferrada and Wagner 2008). Because the fold of an enzyme is essential for its ability to catalyze chemical reactions, a robust fold is more likely to preserve this ability in the face of mutations. The robustness of an enzyme's fold can be estimated through at least 2 complementary approaches. The first determines the number of amino acid changes that a fold has accumulated in its evolutionary history—more robust folds tolerate more such changes, and their amino acid sequences thus change more rapidly in evolution. The second determines robustness directly from the contact density matrix of the fold, which is a descriptor of the amino acid contacts that occur in the fold (England and Shakhnovich 2003; Shakhnovich et al. 2005). As a measure of evolvability, one can estimate the number of different biochemical or biological functions that enzymes with a given fold have evolved since their evolutionary origin, for example, by examining all chemical reactions that are catalyzed by such enzymes. Such an analysis, conducted for 112 ancient enzymes, shows that enzymes with highly robust

folds have evolved more diverse biochemical and biological functions (Ferrada and Wagner 2008).

Broad analyses of many proteins like this one are also supported by more focused studies of individual proteins, such as steroid hormone receptors (McKeown et al. 2014; Starr et al. 2017). These are transcription factors that bind DNA and regulate gene expression in response to steroid hormones. They are ancient proteins whose most recent common ancestor dates to more than 450 million years ago (Eick et al. 2012). This ancestor bound DNA sequences known as estrogen responsive elements (EREs), which mediate gene regulation by estrogen. The ancestor duplicated, and the duplicate evolved the ability to bind specifically to steroid-responsive elements (SREs), which differ from EREs and mediate regulation by different steroid hormones. A combination of phylogenetic analysis, mutant engineering, and biochemical experiments showed that 11 mutations in the duplicated receptor were crucial for the evolution of this new regulatory phenotype (Starr et al. 2017). These mutations occurred outside the DNA-binding domain of the protein. They left the binding specificity of the receptor unchanged, but they changed the general affinity of the receptor to DNA. In doing so, they also increased the mutational robustness of the receptor's ability to bind DNA. As a consequence, they increased by more than 20-fold the proportion of further receptor mutations that bind SREs. For example, among 160,000 variants of the ancestral receptor that lacked these 11 robustness-enhancing mutations, only 41 specifically bound SREs. In contrast, among 160,000 variants of the protein with the 11 mutations, 829 specifically bound SREs, and these variants could be reached by a smaller number of individual amino acid changes (Starr et al. 2017). In sum, mutations that increased robustness also increase the evolvability of this TF's new DNA binding and gene regulatory phenotype.

A biochemical explanation for the positive relationship between robustness and evolvability exists for an unrelated and somewhat more anecdotal example. It involves the zinc finger domain, a protein fold that is part of many TFs and helps them bind specific DNA sequences. The zinc finger domain is exceptionally robust to amino acid changes. For example, all but 7 of its 26 amino acids can be replaced by alanine without destroying its 3-dimensional structure (Michael et al. 1992). In part because of this robustness, zinc finger domains can be engineered toward a great variety of DNA binding specificities (Durai et al. 2005). They are also the most abundant protein domains in the human proteome, occurring in 500 different proteins (Venter et al. 2001). Zinc finger domains fall into different classes. One of them is the C2H2 zinc finger, so named because it contains 2 cysteines and 2 histidines. This motif has evolved much greater DNA binding diversity in metazoans than in other eukaryotes, which can be explained by the greater robustness of the metazoan C2H2 zinc finger. To see why, it is useful to know that the DNA binding affinity of a TF can be determined both by amino acids that contact specific bases and by amino acids that contact the DNA backbone. In non-metazoan C2H2 zinc fingers, DNA affinity is determined by base-contacting amino acids. However, in metazoan C2H2 zinc fingers, affinity is partly determined by backbone contacts. As a result, individual base-contacting amino acids are free to vary without complete loss of DNA binding, which facilitates variation in these amino acids and thus allows variation in DNA binding specificity. In other words, DNA binding is more robust to amino acid changes in metazoans, which also allows DNA binding specificity to vary more broadly (Najafabadi et al. 2017).

In sum, empirical evidence ranging from macromolecules to whole organisms and viruses support the notion that robustness can facilitate the adaptive evolution of new phenotypes on both short and long evolutionary time scales.

11.4 Evolution of Robustness and Evolvability

Robustness can itself evolve, and so can the associated evolvability. If this is the case, the ability of a biological system itself may be an evolving property. I will next discuss empirical evidence that speaks to this possibility.

The general question of whether evolvability itself evolves has recently been reviewed elsewhere (Payne and Wagner 2019). I will thus view this question here in the context of the evolution of robustness. The question can be subdivided into 3 parts. First, *can* robustness (and the associated evolvability) evolve? In other words, is there heritable genetic variation for these properties? Second, *do* they evolve, either in laboratory evolution experiments, or in nature? Third, do they evolve adaptively? That is, can they provide a sufficiently strong benefit that their evolution is driven by this benefit?

The first question is easy to answer. Robustness can evolve. It is subject to heritable variation on all levels of biological organization, from macromolecules and their interactions to whole organisms (Lynch and Conery 2000; Jiménez et al. 2013; Keane et al. 2014; Payne and Wagner 2014, 2019; Najafabadi et al. 2017; Starr et al. 2017; Payne et al. 2018). Several examples come from experiments to engineer specific amino acids into enzymes to increase their robustness (Giver et al. 1998; Salazar et al. 2003; Bloom et al. 2006; Fasan et al. 2008). Likewise in nature, past amino acid changes have increased the robustness of the steroid hormone receptors discussed in section 11.3 (Starr et al. 2017). I also discussed that zinc finger TFs vary between metazoan and other eukaryotes in how they contact DNA, which causes differences in the robustness of their DNA affinity to mutations (Najafabadi et al. 2017). Unrelated examples that I did not discuss include the bacterial transcription factor LexA, whose ability to regulate gene expression can be more or less robust to DNA mutations, depending on whether LexA negatively autoregulates its own expression (Marciano et al. 2014). On a higher level of biological organization, the ability of bacteriophage φ6 to survive and reproduce can vary in its robustness to DNA mutations (McBride et al. 2008). Gene duplications can increase the robustness of an organism to mutations in the duplicated genes (Lynch and Conery 2000), which is associated with increased evolvability in organisms as different as flowering plants (Theissen et al. 1996; Irish and Litt 2005; Hernandez-Hernandez et al. 2007) and vertebrates (Carroll et al. 2001; Olson 2006).

These and other examples also answer the second question: Robustness does evolve (Montville et al. 2005; Borenstein and Ruppin 2006; Bloom et al. 2007; McBride et al. 2008; Zheng et al. 2020). Experimental evolution has increased the mutational robustness of proteins, such as cytochrome P450 (Bloom et al. 2007) and yellow fluorescent protein (Zheng et al. 2020). It also succeeded in increasing the robustness of bacteriophage φ6 (Montville et al. 2005; McBride et al. 2008) and of vesicular stomatitis virus (Codoñer et al. 2006; Sanjuán et al. 2007). More importantly, evolution in the wild has also changed the robustness of various systems. For example, in their distant evolutionary history, steroid hormone receptors have accrued mutations that increase the robustness of their ability

to bind DNA (Starr et al. 2017). More generally, the robustness of a protein's fold tends to increase with the evolutionary age of the protein (Toll-Riera et al. 2012). Stabilizing selection on yeast gene expression has increased the robustness of gene expression to mutations (Vaishnav et al. 2021). Many gene duplications in eukaryotic genomes have increased the robustness of a gene's function to mutations (Lynch and Conery 2000).

The third question regards the forces that drive the evolution of robustness and the associated evolvability. This question does not have a single answer. It depends on the kind of evolving system and on the conditions of its evolution, such as its population size and the mutation rate. For example, mutations are usually rare and therefore do not cause strong selection pressure for increased robustness to mutations. As a consequence, theory predicts that mutational robustness can evolve as an adaptation to mutations only when mutations are sufficiently frequent or when populations are sufficiently large (G. Wagner et al. 1997; van Nimwegen et al. 1999; De Visser et al. 2003; Wagner 2005). When these conditions are met, however, it has been shown that experimental evolution can readily increase robustness to mutations as an adaptation to mutations itself, both in proteins (Bloom et al. 2007) and in RNA viruses (Codoñer et al. 2006; Sanjuán et al. 2007).

Robustness to mutations can also evolve for at least two other reasons. First, it can emerge as a by-product of robustness to environmental change. Robustness to environmental change often entails robustness to mutations (Ancel and Fontana 2000; Meiklejohn and Hartl 2002; Bloom et al. 2006; Domingo-Calap et al. 2010; Butković et al. 2020), and environmental change is usually much more frequent than DNA mutation. As a consequence, populations experience stronger selection to become robust to environmental change (G. Wagner et al. 1997; Meiklejohn and Hartl 2002; Wagner 2005). Second, robustness may sometimes increase for no adaptive reason at all. For example, robustness often increases at least temporarily after a gene duplication, because 2 duplicate gene copies are usually redundant, such that the second copy can compensate for a deleterious mutation in the first copy (Lynch and Conery 2000). Gene duplications are frequent by-products of DNA repair and recombination processes that cells need to maintain their genomic integrity. They can be adaptive (Conant and Wolfe 2008; Nasvall et al. 2012), but they may also be maladaptive, because they carry a cost in terms of the energy needed to express them (Wagner 2007; Lynch and Marinov 2015). Thus, a gene duplication may entail high genetic robustness without necessarily being adaptive.

Such evidence shows that robustness evolves, but it does not answer the question of whether robustness can evolve because it facilitates evolvability. The problem is that evolvability, much like robustness, is a dispositional trait—it affects the potential for future evolution but need not convey immediate benefits to an organism. This indirect benefit of evolvability suggests that selection favoring evolvability is weaker than selection for other traits with direct benefits.

Such indirect selection, however, can still enhance evolvability, as a recent evolution experiment shows (Zheng et al. 2020). In this experiment, we studied the evolvability of the phenotype green fluorescence from the ancestral phenotype of yellow fluorescence in a population of evolving fluorescent proteins. More specifically, the experiment consisted of 2 separate phases. In the first phase, we evolved populations of yellow fluorescent protein toward increased *yellow* fluorescence during 4 generations of random mutation and directional selection. We subjected 4 populations to strong directional selection for

this ancestral phenotype, 4 populations to weak directional selection, and 4 populations to no selection. After these 4 generations, we subjected each of the 12 populations to 4 more generations of equally strong selection for *green* fluorescence. We found that the populations that had been under strong selection for the ancestral yellow phenotype evolved green fluorescence more rapidly and to a higher level than did the other populatons. A combination of high-throughput population sequencing and mutant engineering showed that they accumulated mutations that increased mutational robustness by increasing the protein's foldability—the ability to form a correctly folded protein. These same mutations also increased protein fitness, but they did so predominantly through their effect on robustness, which hindered deleterious mutations from slowing down adaptive evolution, and which facilitated the spreading of mutations beneficial for the new phenotype (Zheng et al. 2020). This experiment shows that under the right conditions, most notably strong selection and a sufficiently high mutation rate, mutational robustness can evolve adaptively, because it enhances evolvability. An important task for future work is to find out whether such adaptive evolution of evolvability mediated by robustness also exists in the wild.

11.5 Summary and Outlook

In sum, a growing body of experimental evidence shows that robustness can facilitate evolvability. What is more, robustness can and does evolve, and it can even evolve adaptively to enhance evolvability. Genotype networks provide a unifying framework that can help explain experiments like those described in this chapter. This framework can help us understand that mutational robustness can facilitate evolvability, because it allows evolving populations to explore a wider region of a genotype space, in which the chances of finding novel and adaptive phenotypes are greater than in a smaller region.

Some of these experiments also illustrate the limitations of the genotype network framework in its simple form sketched in figure 11.1c. For example, the framework abstracts phenotypes into categories, which allows an enumeration of novel phenotypes for the purpose of mathematical and computational analyses (Schuster et al. 1994; Reidys et al. 1997; Ciliberti et al. 2007; Rodrigues and Wagner 2009). However, many phenotypes that are amenable to experimentation are continuous quantities, such as increased antibiotic resistance or enzyme activity. Thus, any one genotype network is embedded in an adaptive landscape, where genotypes with the same qualitative phenotype need not be neutral in fitness. They may differ quantitatively in the phenotype they encode, which means that this landscape's topography affects their evolutionary fate and their ability to discover novel phenotypes. To understand how robustness affects evolvability through its evolutionary dynamics on an adaptive landscape remains an exciting task for future theoretical work.

An additional complication is that many genotypes encode multiple phenotypes, even at thelowest levels of biological organization, where a promiscuous enzyme can catalyze multiple biochemical reactions (O'Brien and Herschlag 1999; Khersonsky and Tawfik 2010; Wagner 2014). An evolving genotype may thus encode an entire spectrum of phenotypes that may change with each step of evolution on and near a genotype network. Thus, although the framework of figure 11.1c serves to communicate a key principle, expanding and adapting it for different purposes will be essential to understanding why robustness often facilitates evolvability in the complex world of biological evolution.

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