

# Robustness and Evolvability in Living Systems

Andreas Wagner

Princeton Studies in Complexity

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## **ROBUSTNESS AND EVOLVABILITY IN LIVING SYSTEMS**

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*Andreas Wagner*

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**For Elisabeth and Lani Wagner**

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## **ROBUSTNESS AND EVOLVABILITY IN LIVING SYSTEMS**



# 1

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## Introduction

Living things are unimaginably complex, yet they have withstood a withering assault of harmful influences over several billion years. These influences include cataclysmic changes in the environment, as well as a constant barrage of internal mutations. And not only has life survived, it has thrived and radiated into millions of diverse species. Such resilience may be surprising, because complexity suggests fragility. If you have ever built a house of cards, you will know what I mean: The house eventually comes tumbling down. Why is an organism not a molecular house of cards? Why do not slight disturbances (especially genetic disturbances in the form of mutations) cause key organismal functions to fail catastrophically? And is the robustness of organisms to change itself a consequence of past evolution? How does it affect evolvability, the potential for future evolution? These are some of the key questions I will address here.

A biological system is robust if it continues to function in the face of perturbations. This is the working definition of robustness I use in this book. The perturbations can be genetic, that is, mutations, or nongenetic, for example, environmental change. A variety of other names—buffering, canalization, developmental stability, efficiency, homeorhesis, tolerance, etc. (171, 183, 186, 368, 472, 499, 578)—have been used for the same phenomenon, and my choice of one among them is arbitrary. The above definition implies that one can sensibly discuss robustness only if one has clarity about two cardinal questions: What feature of a living thing is robust? And what kind of change is this feature robust to?

With respect to the first of these cardinal questions, it is clear that ultimately robustness of only one organismal feature matters: fitness—the ability to survive and reproduce. However, fitness is hard to define rigorously and even more difficult to measure. In addition, a change in fitness can have many different causes. For instance, a mutation that blocks a chemical reaction in metabolism affects fitness for different reasons than a mutation blocking embryonic development. An examination of fitness and its robustness alone would thus not yield much insight into the opening questions. Instead, it is necessary to analyze, on all levels of organization, the systems that constitute an organism, and that sustain its life. I define such systems loosely as assemblies of parts that carry out well-defined

biological functions. Examples include DNA with its nucleotide parts, proteins with their amino acids, metabolic pathways and their enzymes, genetic networks and their genes, and developing organs or embryos with their interacting cells. A good part of this book surveys what we know about the robustness of biological systems on multiple levels of biological organization.

With respect to the second cardinal question, what are organisms robust to, this book has a restricted scope: It focuses on robustness to genetic change. I will call this kind of robustness *genetic robustness* or *mutational robustness*. This focus has three motivations. First, genetic change has more serious consequences than nongenetic change. A genetic change is a permanent alteration in the “wiring” of a biological system, and its effects, or lack thereof, thus deserve special attention. Second, by and large, mostly genetic change is heritable, and thus has much more serious long-term consequences on organismal lineage than nongenetic change. Thirdly, a comprehensive account of robustness against nongenetic change would be daunting. For instance, an exhaustive treatment of robustness to environmental change would have to include just about all homeostatic phenomena in biology. These phenomena include the regulation of osmotic balance, metabolite concentrations, and gene expression, thermo-regulation in endotherm organisms, flight stabilization in birds, and on and on. The literature on many of these phenomena is already large and needs no further addition. Robustness to mutations, on the other hand, has not been as comprehensively studied. In addition, it is a well-defined phenomenon where a search for general principles that unify observations on different levels of organization is easier. I will propose some such principles here. All this is, of course, not to say that robustness to nongenetic change is unimportant. In fact, it is associated with mutational robustness and may be very important for the evolution of such robustness, as I argue in chapter 17.

## Why Study Robustness?

The first and most important reason to study robustness is already stated in the opening paragraph: Why can unimaginably complex systems withstand so much change? As we shall see, biological systems are indeed robust on all levels of organization. Proteins can tolerate thousands of amino acid changes, metabolic networks continue to sustain life even after removal of important chemical reactions, gene regulation networks continue to function after alteration of key gene interactions, and radical transformations in embryonic development can lead to an essentially unchanged adult organism.

A second reason to study robustness (an evolutionary biologist's reason) derives from the fact that evolution by natural selection requires variation among organisms that reflects genetic variation. Genetic variation is abundant in most species, yet how it translates into phenotypic variation is still unknown. In the second part of the 20th century, a debate about precisely this question dominated evolutionary biology. This debate focused on the role and abundance of neutral mutations, mutations that do not affect the function of a biological system. The more neutral mutations a biological system allows, the greater is its mutational robustness, and mutational robustness thus has an important role to play in this debate. Mutational robustness influences the extent to which genetic variation, the result of past mutations, is translated into phenotypic variation. Even more importantly, if mutational robustness itself is subject to evolutionary change, then the ability to evolve by natural selection evolves, and thus evolvability evolves. For this and other reasons, neutral mutations will play a central role in this book. I will argue that they may play a very important role in promoting evolutionary innovation.

The third reason to study robustness regards engineering principles of robust systems. Is robustness in the living fundamentally similar and different from robustness in engineered systems? Can human engineers learn from robustness in the living? Only an engineer could be the judge, but the many examples scattered throughout the book may help in making this judgment. Although the book is primarily directed toward biological systems, I devote one short chapter to robustness in engineered systems.

## How to Study Robustness

Empirical evidence for robustness comes in two different forms. First, one can perturb a part of an organism (a protein), a trait (wing shape), or a capability (amino acid biosynthesis) through mutations. The less the feature's properties change in the face of perturbation, the more robust it is. The second type of evidence relies on naturally occurring perturbations, mutations that occurred in evolutionary history. That is, one can compare closely related species that have the same trait or capability, and examine whether they achieve it by different means. If so, this indicates robustness, because not only can the same feature be designed in different ways, these different ways originated in a recent common ancestor and are thus reachable from each other by mutation or recombination. As with most applications of the comparative method, the results of this second approach are more tentative than the results of systematic perturbations.

Neither kind of evidence is easy to produce. Many biological systems, from macromolecules to genetic networks, have large numbers of parts

that can occur in many configurations. To assess their robustness systematically requires many perturbations and subsequent measurements of system properties. For instance, to explore only a few variants at each amino acid positions of a protein, one needs to generate thousands of mutant proteins and measure their activity. The evolutionary approach to study robustness suffers from a related problem. First, to compare different organisms is to analyze only a few end products of many possible paths evolution could have taken. Second, sometimes even that is infeasible. There are preciously few well-studied organisms for which any one biological process above the gene level is well characterized, because such characterization is time-consuming. For instance, it took thousands of man-years to elucidate the structure of the genetic network responsible for segmenting a fruit fly's body. It would be prohibitive to analyze the same network in many related species to determine how much its structure has changed while leaving its function intact.

In sum, the experimental evidence to assess robustness in biological systems is hard to come by. The problem is partly alleviated by modeling of such systems, using both analytical and computational methods. Quantitative models that are based on experimental information can provide accurate predictions about a system's robustness, even when systematic perturbations or evolutionary comparisons are difficult. Many of the case studies below involve a tight integration between experimental evidence and quantitative modeling. Some of the most intriguing questions, such as whether robustness itself can evolve, have been mostly addressed with computational models. The heavy reliance on modeling to understand biological robustness may change as more experimental data accumulates. However, because of the many difficulties of providing such data, quantitative modeling will always play an important role in understanding the robustness of biological systems.

## An Emphasis on Mechanism

One can analyze biological systems, their robustness, and its evolution from two very different perspectives. The first of them, exemplified by biochemistry and molecular biology, emphasizes mechanistic understanding, dissection of systems and their parts. Most of this book emphasizes this mechanistic perspective. A second approach is represented by population genetics and, even more so, by quantitative genetics. These disciplines emphasize the statistical effects of genes on fitness rather than the roles of genes in a molecular machinery. Both disciplines provide important perspectives complementary to those of molecular biology (55, 104, 141, 185, 186, 228, 244, 274, 305, 404, 415, 448, 462, 472, 499, 519, 520,

562, 579, 582, 585, 591, 592, 610). Population genetics, for example, identifies the conditions—selection pressures, mutation rates, population sizes, etc.—under which robustness can evolve, which is completely outside the scope of molecular biology. I have included general population genetic insights into the evolution of robustness. Nonetheless, the book contains comparatively little material from population genetics and next to none from quantitative genetics. The main reason is the following.

Population genetics and quantitative genetics have been very successful partly because they have eliminated the mechanistic details of biological systems from their thinking. However, the elimination of such detail and the resulting phenomenological perspective on organisms come at a price: Evolutionary explanations built on a statistical understanding of gene effects may be difficult to interpret. Take a recent example from a growing literature on how to measure robustness with quantitative genetic methods (244). Suppose you had found that during the evolution of an organismal lineage B from some ancestral lineage A, the mutational robustness of some trait, say the length of a fly's wing, has apparently increased. That is, the trait shows less change in response to the same amount of "mutation pressure" in lineage B than in lineage A. Houle pointed out that such apparent differences in robustness among lineages and traits could be caused by differences in the genome target size of these traits (244). The genome target size is the number of genes contributing to a trait. In other words, a trait's robustness may appear increased merely because the number of genes contributing to it decreased. To estimate this genome target size with the methods of quantitative genetics is difficult, partly because many genes with very subtle statistical effects contribute to most traits. Because quantitative genetics has not yet resolved such fundamental problems, I chose to focus here on systems whose inner workings are understood to some extent.

## Principles of Robustness

This book could not have been written 15 years ago, because much of the mechanistic information I emphasize here has accumulated only recently. One consequence of this fact is that this field of research is not mature. It is rife with open questions, yes, dominated by open questions, questions that define entire research programs in systems biology. (I summarize some of these questions in the short epilogue.) This observation points to two motivations to write this book now. First, a survey of our knowledge brings our ignorance into sharp relief. Second, the available pieces of the puzzle enable us to see the outline of the whole, and allow us to make some general statements about it. Beyond the presentation of the

evidence, you will thus find many informed guesses at the shape of the whole here. Whether or not there will be a unified theory of robustness in biological systems, some unifying principles will emerge once this field has reached maturity. Here is a brief summary of a few such principles (my credo, if you will), principles that later chapters elaborate in much greater detail and with concrete examples.

*Most problems the living have solved have an astronomical number of equivalent solutions, which can be thought of as existing in a vast neutral space (chapter 13).* A neutral space is a collection of equivalent solutions to the same biological problem. Such solutions are embodied in biological systems that ensure an organism's survival and reproduction. Both direct perturbation studies and indirect comparative studies support the notion that problems with many solutions are the rule rather than the exception. This holds on multiple levels of biological organization. We see it, for example, in the structure of important macromolecules such as proteins and RNA, where there are astronomically many different ways to build a molecule with a given structure and function. We see it also in the architecture of transcriptional regulatory regions, which can change drastically in evolution without any change in function. We see it in the structure of metabolic and genetic networks, where large changes in network structure can have negligible effects on network function in any one environment. We even see hints of it at the highest level of organismal organization, where radically different pathways of embryonic development may lead to essentially unchanged adult organisms.

*Biological systems are mutationally robust for two reasons. First, robust systems are easier to find in the blindly groping search of biological evolution, simply because of the large neutral space associated with them (chapter 13).* In other words, robust systems are systems with a large associated neutral space of equivalent solutions to a given problem. Such systems are easiest to discover in evolution, because they represent a large proportion of all possible solution. Their robustness results from the structure of neutral spaces itself, and may be independent of the particular circumstances under which an organism or the system evolved, such as population sizes or mutation rates. *Second, natural selection can further increase robustness by incremental evolution of a system within a neutral space (chapters 13, 16, 17).* Neutral spaces are not homogeneous. We know this from studies of the neutral spaces associated with the structure of biological macromolecules, and to a more limited extent from studies of genetic networks and the genetic code. This means that neutral spaces often have regions characterized by greater robustness, where mutations are less likely to change a system's structure or function, and regions of lesser robustness. Regions of lesser robustness are more sparsely populated with systems that perform a given function. Evolution by natural selection can

drive an evolving population toward regions of a neutral space with high robustness.

*Either mutations or nongenetic change can drive incremental evolution of mutational robustness (chapters 16, 17).* It is at first sight obvious that robustness to mutations could be an adaptation to mutations. However, mutations are rare in most organisms. This implies, as I argue in chapter 16, that the conditions under which mutations can cause an increase in robustness are very restrictive. They require large populations or high mutation rates. Systems robust to mutations, however, are also robust to nongenetic change. Thus, mutational robustness can emerge as a by-product of selection for robustness to nongenetic change. This second mechanism for incremental evolution of robustness is much less restrictive, because organisms are constantly exposed to a barrage of nongenetic change.

Both of these explanations rely only on individual-based selection, and not on group, lineage, or species selection. That is, robustness need not be advantageous to a group of cells or organisms to increase in evolution. The main reason to emphasize individual-based selection is not so much that group selection is controversial and that it may occur only under limited conditions. Rather, almost all features of organisms that are hard to explain otherwise—among them altruism, sex, and evolvability itself—are easy to explain using group selection. The real challenge is to explain the evolution of robustness and evolvability through individual-based selection, which we know is ubiquitous.

*Robustness and neutral mutations are key to evolutionary innovation (chapter 14).* Robust biological systems permit many neutral mutations, mutations that do not affect a specific system function. However, these mutations can affect other properties of the system, properties that may be the source of future detriment or benefit, and also the source of evolutionary innovations. Much like there are few mutations that will affect the phenotype under all circumstances—in all environments or genetic backgrounds—there are no mutations that are neutral under all circumstances. As I argue in chapter 14, if we can abandon an essentialist concept of neutrality—once neutral, always neutral—the concept of neutrality will continue to be useful and provide insight into the mechanics of innovation.

*Redundancy of a system's parts is a minor mechanistic cause of robustness to mutation. More important is distributed robustness (chapter 15).* In distributed robustness, interactions of multiple system parts, each with a different role, can compensate for the effects of mutations. I use the word redundancy here only for two or more system parts that perform the same or similar tasks. Perhaps the best example is gene redundancy. Gene redundancy occurs if one gene has several copies in a genome. Such redundancy can render an organism robust to mutations in one of these copies. I argue in chapter 15 that redundancy plays a role in mutational

robustness, but not the predominant role. This holds for systems whose parts are genes, but also for systems on other levels of biological organization, such as biological macromolecules, whose parts are nucleotides or amino acids.

*Fragility in a biological system, the opposite of robustness, can have several evolutionary causes (chapter 18).* By fragility I mean that a system varies greatly in either structure or function in response to mutations. The first possible cause is that the biological problem to which the system is a solution has only a few alternative solutions and thus a small associated neutral space. Second, variation in the system may be advantageous to the organism. A paradigmatic example of advantageous variation is antibody diversity in the vertebrate immune system. The third possibility is that trade-offs with other aspects of the system's function preclude maximal robustness. Potential examples include enzymes: To catalyze chemical reactions, enzymes need to change their tertiary structure, their folded three-dimensional structure, in subtle ways. Such flexibility is not possible if this tertiary structure is maximally robust to mutations, as I discuss in chapter 5.

*Many natural systems below and beyond living organisms show great robustness to changes in their parts. Such robustness can also increase over time, but the cause is usually self-organization instead of natural selection (chapter 19).* I will illustrate this principle with one main example, the robustness of ecological communities to species invasions. (Ecological communities, although composed of living things, are strictly speaking not themselves living things.) The example is my only excursion into ecology, where the subject of robustness to various perturbations has been of long-standing interest (362), and has spawned a bewildering array of terminology and different criteria for robustness (212). *Many of the mechanistic principles that underlie robustness in living systems can also be observed in man-made, engineered systems (chapter 20).* I will illustrate this notion with some anecdotal examples from areas such as telecommunications and electrical engineering. A more exhaustive comparison would itself merit a book.

### A Word on the History

It has been said that nothing is ever new. To the extent that this is true, it also holds for these and other ideas I emphasize here. Some of them have been expressed previously, in various degrees of clarity, within a restricted field of investigation or with an emphasis different from that on evolution I take here (130, 183, 201, 491). I have become aware of some previous work only late during this writing, and may be completely unaware of

other work. Germs of these concepts may go back many decades and may be buried, unbeknownst to me, in one or the other parenthetical observation of many research papers. However, there are two main historic threads of research into mutational robustness. Both go back to the first half of the 20th century. The first of them regards the phenomenon of *dominance*. Dominance means that a phenotypic feature of an organism is robust to elimination of one among two copies of a gene product or to a corresponding 50% change in the concentration of a gene product. The phenomenon has been known for most of the 20th century, and its discovery can be traced back to Gregor Mendel's experiments in the 19th century, which are cornerstones of classical genetics (364). A major protagonist in the history of research on dominance is Ronald Fisher (155, 156), who proposed the first evolutionary explanation of dominance in the 1930s. I discuss this explanation in chapter 8, as well as some reasons why most evolutionary biologists consider it no longer viable.

A second important phenomenon and early line of investigation is that of *canalization*. An organismal feature is canalized if its embryonic development is insensitive to variation in the environment or in genes. The term canalization originated with the embryologist Conrad Waddington (575, 578). He and others studied canalization by trying to disrupt it in organisms such as fruit flies and in mice through environmental stressors or specific mutations. The result of disrupting canalization is a drastic increase in variation that is caused by previously hidden genetic variation. Canalization is thus a specific aspect of robustness in organismal traits. I revisit this phenomenon and some of its history in chapter 11.

Research on dominance and canalization constitute the two main lines of investigation into mutational robustness. However, I note in passing that the broader phenomenon of robustness to any change, whether genetic or nongenetic, has a much longer history. A case in point is the concept of homeostasis, an organism's ability to sustain a physiological state in the face of change. It was coined in 1932 by the American physiologist Walter Cannon. However, its roots can be traced back to the French physiologist Claude Bernard and his book *Introduction to the Study of Experimental Medicine* (44). In essence, Bernard argued there that the constancy of the milieu inside an organism results from regulatory mechanisms inside the body.

## Functions and Purpose, Problems and Solutions

As the preceding pages show, I will heavily use functional language in this book. That is, I will speak of biological systems as serving specific functions or purposes inside an organism. In other words, such systems solve

problems that organisms face in reproducing and surviving. For instance, enzymes are systems that solve the problem of converting chemical compounds into useful forms; gene circuits in development solve the problem of reliably patterning an embryo to produce a viable adult; the genetic code solves the problem of translating genetic information into a protein's amino acid sequence; and so on.

Such language raises thorny philosophical problems if taken literally (470, 631). Part of the reason is that words like “function” and “problem” insinuate an intelligent agent standing behind a system’s design. However, for all we know, the biological systems I examine here emerged from the blindly groping search that characterizes all of biological evolution. That they embody solutions to important biological problems is obvious only in hindsight, after the systems that embody these solutions survive. It should be understood that functional language merely provides a convenient and compact way to describe the endpoint of the convoluted paths evolution takes.

## Who Is This Book for?

If you are a specialist who already knows some or most of the literature in this field, much of this book will not be news to you. However, if you are a nonspecialist interested in the questions I pose, this book may be for you. The book presupposes some knowledge of biological principles, particularly genetics and biochemistry, on the level of an introductory course in both subjects. Most of the chapters of parts I and II survey our knowledge in a representative range of examples, and the relevant background material is contained within each chapter. Only a few chapters contain mathematical material, which requires some basic understanding of linear algebra, differential calculus, probability theory, and differential equations. However, even where I found some mathematical treatment necessary, such as in the chapters on metabolism, I took pains to describe the central concepts verbally as well.

## The Organization of This Book

The book consists of four parts. The chapters of parts I and II take you on a tour through examples of genetic robustness. Each chapter addresses a different aspect of the cardinal question “what is robust?” In other words, each chapter examines the robustness of a different feature of an organism. The sequence of chapters is a tour through the hierarchy of biological organization, from molecules to whole organism. Some of the

central ideas I just mentioned make an appearance in these chapters, but I defer their detailed discussion to part III.

Chapter 2 examines the robustness of the genetic alphabet to replication errors. It is one of the most speculative chapters, because it examines evidence for several possible DNA and RNA chemistries, among which only one is realized in life as we know it. Chapter 3 discusses a large body of work on the robustness of the genetic code to nucleotide changes in individual codons. Chapter 4 examines the robustness of RNA structure to nucleotide changes, and chapter 5 does the same for protein structure and amino acid changes. Chapter 6 focuses on the robustness of proteins to a different kind of genetic change, recombination. It also briefly discusses a phenomenon related to recombination, lateral gene transfer, which has profoundly influenced microbial evolution.

The subsequent chapters constitute part II and discuss higher levels of biological organization. Chapter 7 discusses how gene expression can be robust to changes in promoter organization. Chapters 8 and 9 survey the robustness of metabolic pathways and metabolic networks to changes in enzyme activity. Chapter 10 examines the robustness of genetic networks in embryonic development to changes in regulatory gene interactions. Chapter 11 focuses on the organismal level and on phenotypic characters, such as the wings and eyes of insects. It surveys evidence that the embryonic development of such characters is highly robust to mutations, and how this robustness can be disrupted in laboratory experiments. In addition, the chapter also discusses the breakdown of robustness in genetic diseases, a phenomenon illustrating the importance of robustness for medicine. Chapter 12 uses examples from the evolution of three very different organisms, nematode worms, sea urchins, and parasitic wasps, to illustrate how the formation of whole body plans can be robust to massive developmental changes, which are ultimately caused by genetic change.

The chapters of parts I and II contain a moderate number of illustrative, hand-picked examples. I chose these examples either because they are especially well understood or because they illustrate a general principle well. This means that the material I discuss is representative rather than comprehensive. It contains obvious omissions that I made for one or the other reason. One such example regards neural circuits and the computations they perform. Theoretical work shows that neural circuits can be highly robust to removal of neurons and to removal of interactions between neurons (19). However, we know little about the robustness of biological neural circuits—as opposed to abstract models of such circuits—to mutations.

Part III uses many of the examples in parts I and II as raw material to discuss general principles behind robustness and its evolution. The first chapter in this part, chapter 13, focuses on one of the key concepts emerging

from parts I and II, the concept of a neutral space. Chapter 14 explores the relation of robustness to future evolutionary potential—evolvability. It emphasizes the positive role that robustness and neutral mutations play for evolvability. Chapter 15 focuses on the phenomenon of redundancy. The chapter discusses empirical evidence that distributed robustness and not redundancy of parts may be the predominant mechanistic cause of robustness in biological systems. Chapters 16 and 17 focus on the evolution of mutational robustness. Specifically, chapter 16 discusses how robustness can evolve as an adaptation to mutational pressure. Chapter 17 shows that mutational robustness can emerge as a by-product of selection for robustness to nongenetic change. Chapter 18 focuses on systems that are not robust but fragile. It discusses various evolutionary causes of such fragility.

Part IV is an elaborate afterthought that relates mutational robustness in the living to robustness in other systems. Chapter 19 shows how robustness in natural inanimate systems can change over time. It does so through a mechanism fundamentally different from natural selection, a mechanism involving self-organization. Chapter 20, finally, provides a nexus between robustness in the living and in nonliving engineered systems. It highlights some similarities between these kinds of systems, such as the existence of distributed robustness. The book closes with an epilogue that states important open questions about robustness and its evolution.

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## **Part I**

### **ROBUSTNESS BELOW THE GENE LEVEL**



# 2

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## The Genetic Alphabet

What is robust? The sequence of letters in RNA or DNA.

What is it robust to? Replication errors.

Why does the genetic material have four letters, and why are they exactly the four letters we know: A, T(U), C, and G? One might think that there is no other way to ensure accurate replication. However, that is far from certain, as we will see here.

Because we do not know of any organisms that have genetic material with a different alphabet, this chapter contains a fair dose of speculation, but I included it for two reasons. First, it illustrates that a careful look may reveal robustness—and possibly traces of its evolution—even on the lowest level of biological organization, that of genetic material. Second, this chapter speaks to a distinction that will later become important. It is the distinction between robustness to genetic changes on one hand, and robustness against environmental changes and random events (noise) on the other hand. In many of the systems I will discuss later, genetic change is clearly distinguishable from environmental change. However, this distinction breaks down on the level of DNA. To be sure, the misincorporation of nucleotides into genetic material is the reflection of random errors by RNA or DNA polymerases. However, such noise is here *synonymous* with genetic change.

In this chapter, I first briefly review the basic chemistry of the Watson-Crick base pair. Then I discuss two requirements for a robust genetic alphabet: strong binding between complementary bases and, equally important, repulsion between noncomplementary bases. Next, I discuss some recent work suggesting that more than one genetic alphabet fulfills these requirements (351). Note that faithful replication, albeit my focus here, may not solely determine the nature of our genetic alphabet (180, 537). Other factors include a nucleotide's ability to be processed efficiently by replication enzymes, and differences in catalytic abilities of RNA enzymes that are composed of different nucleotides. Such catalytic abilities may have been especially important in early, RNA-based life. I focus on faithful replication here, however, because it relates most directly to mutational robustness.

Although most of the conclusions of this chapter would apply to DNA, most pertinent work uses RNA (351, 352, 535, 536). Part of the reason is that RNA probably preceded DNA as genetic material. As a result, the evolution of an RNA alphabet has received greater scrutiny.

### The Watson-Crick Base Pair

It is difficult to gauge in how many alternative ways one could design genetic material that uses the complementary principle familiar from DNA. While some alternatives have been proposed (537, 574), others have almost certainly eluded human imagination. However, even within the confines of the Watson-Crick base pair, several possible alphabets might ensure faithful replication. In a Watson-Crick base pair, complementary bases always involve one purine base (A or G) and one pyrimidine base (U or C), and never two pyrimidine or two purine bases.

Complementarity is ensured by up to three hydrogen bonds between the two bases. The chemical structure of a base pair such as G-C is shown in Figure 2.1. Base pairing is mediated by hydrogen bonds at a maximum of three positions between two bases. In each hydrogen bond, one hydrogen atom of an amino group ( $\text{NH}_2$ ) serves as a hydrogen donor, whereas an oxygen atom or a nitrogen atom serves as a hydrogen acceptor. In other words, in each hydrogen bond, the hydrogen of an amino group is paired with either an oxygen atom or a nitrogen atom. In a Watson-Crick base pair, the middle hydrogen bond (as shown in Figure 2.1) always involves two heterocyclic nitrogen atoms—atoms in one of the rings constituting a base—one of which carries a hydrogen atom. The upper and lower hydrogen bonds are mediated by interactions between oxygen atoms (hydrogen acceptors) and amino groups (hydrogen donors). If we focus on only one base, for example, a purine base, we see that its upper position can carry either an oxygen atom or an amino group, and the same holds for the lower position. The middle position always carries a heterocyclic nitrogen atom, either with or without a hydrogen atom. Any purine base thus has a total of eight ( $2 \times 2 \times 2$ ) possible configurations of hydrogen donor–acceptor groups at this base. For the strongest possible base pairing, the pyrimidine base complementary to this purine must contain a hydrogen acceptor group wherever the purine base contains a donor, and vice versa. Thus, any one of the eight possible purine configurations completely determines the corresponding acceptor–donor configuration of the complementary pyrimidine base.

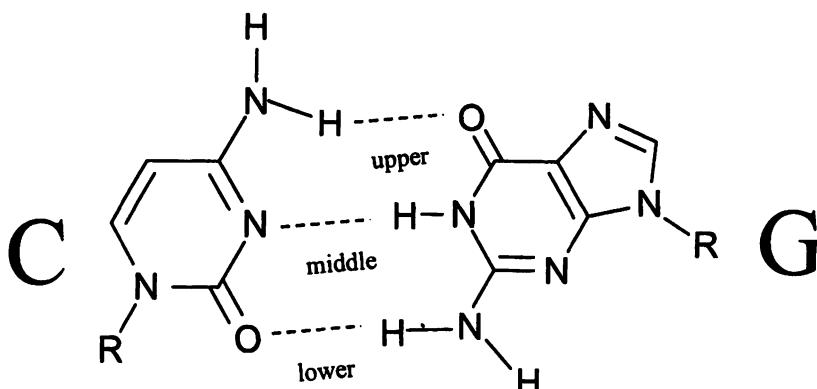
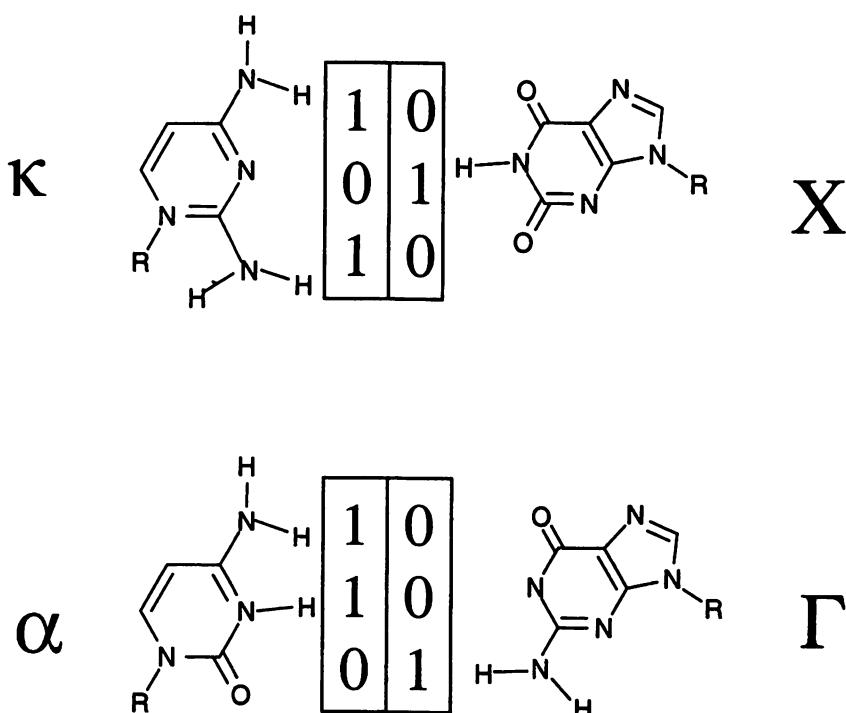


Figure 2.1 The chemical structure of a G-C base pair. The hydrogen bonds are designated as upper, middle, and lower, as in the text.

In sum, there exist eight different Watson-Crick base pairs that ensure strong complementary base pairing. However, in actual genetic material we see only two of these eight pairs. (Whereas one of them, G-C, uses all three hydrogen bonds, the other, A-U, uses only two. The reason is that adenine does not have an exocyclic residue—neither oxygen nor amino group—at the lowest of three candidate positions for hydrogen bonding.) Why do we see exactly the two complementary base pairs G-C and A-U? Part of the answer comes from instabilities in some bases. Such instabilities fall into two categories: chemical instabilities, where a molecule disintegrates under physiological conditions, and tautomeric instabilities, where a molecule can coexist in more than one form with the same composition but different arrangement of its atoms. For example, one of the eight possible bases, known as isoC, is prone to hydrolysis under chemical conditions that allow nucleic acid replication (442). The same holds for a purine or pyrimidine base that has only hydrogen acceptor groups. IsoG, a third base analogue in the group of eight, is unstable due to its coexistence with tautomers. However, even if such unstable bases are excluded, there still remain more than the realized number of candidate base pairs, some of which are shown in Figure 2.2. MacDónaill (351) has pointed out that the absence of such base pairs in nature may be explained if one considers that faithful replication is subject to two conflicting constraints. First, as already mentioned, binding energies of complementary bases must be attractive. Second, binding energies between noncomplementary bases must be repulsive. That is, the lowest possible number of hydrogen donor-acceptor pairs must occur between noncomplementary bases.



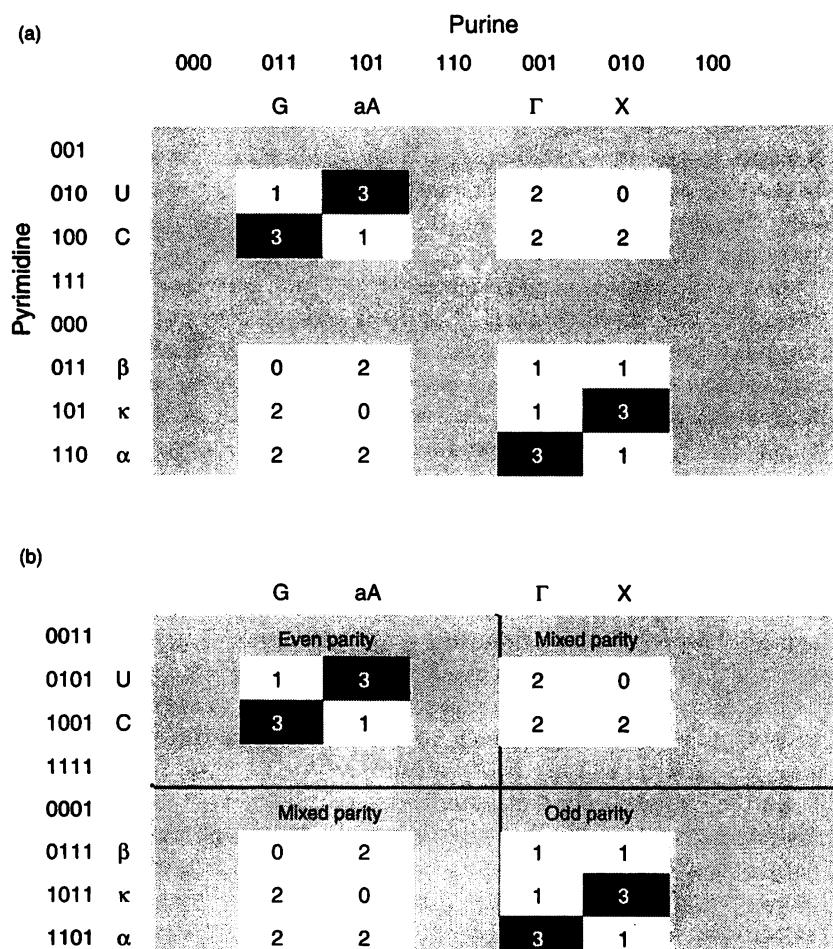
**Figure 2.2** The chemical structure of two base pairs that are not found in nature. The nomenclature is taken from (442, 535). A hydrogen donor group in a base is indicated by a one, a hydrogen acceptor group by a zero.

### Alternative Alphabets with Faithful Pairing

To explore the consequences of these two constraints, MacDónaill uses a binary encoding of the configuration of hydrogen donor groups (Figure 2.3), where the presence of a hydrogen donor group at one of three positions is indicated by a one, and a hydrogen acceptor group by a zero. In this notation, the hydrogen donor–acceptor configuration of nucleotide G becomes (011) and that of C becomes (100). A large—maximally three—number of bits that differ between two bases indicate strong complementarity. Mathematically, this difference is equivalent to the Hamming distance  $H$ , or the Boolean exclusive OR (XOR) function of the binary representations. For instance, the Hamming distance  $H(G,C)$  between G and C equals three. If two bases have a distance different from three in this representation, then they are noncomplementary. That is, they have both hydrogen donors or hydrogen acceptors in one or more

juxtaposed positions. This binary representation of base complementarity is of course a crude abstraction of the chemistry of base pairing. For example, it assumes that the contributions of individual hydrogen bonds to overall binding energy are exactly the same, and that they can simply be added to give the overall energy. While these assumptions do not hold in general, detailed calculations of binding energies between different bases support the notion that their binary distances reflect binding strengths (352).

Figure 2.3a shows an overview of stable base pairs and their pairing strength in this representation. The rows and columns in the figure correspond to purine and pyrimidine bases, respectively. The large gray region reflects unstable bases, or bases complementary to unstable ones. Black entries correspond to bases with the maximum of three hydrogen bonds. The biologically observed bases belong in the upper-left block in the figure. Brief inspection reveals that this block of bases shows superior discrimination between complementary and noncomplementary bases to some combinations of base analogues not observed in nature, such as those shown in the upper-right block involving bases  $\Gamma$  and  $X$ . First, in the block of realized bases, there are only two strongly complementary bases with three opposing hydrogen donor-acceptor combinations ( $G-C$  and  $aA-U$ ). Conversely, the noncomplementary bases have identical donor-acceptor configurations at two out of the three possible positions. The upper-right block contains base pairs, such as  $C-X$ , that have a maximum of only two opposite donor-acceptor configurations. If one accepts such pairs as complementary, then three out of the four possible pairs ( $U-\Gamma$ ,  $C-\Gamma$ ,  $C-X$ ) are complementary, such that the discrimination between complementary and noncomplementary bases is no longer clear-cut. A similar pattern is observed for the lower-left block of nucleotides. In contrast, the lower-right block of nucleotides has two strongly complementary base pairs ( $\kappa-X$ ,  $\alpha-\Gamma$ ), and equally good discrimination between all noncomplementary bases. The noncomplementary bases have identical hydrogen donor configurations at two out of three positions, just as for the biologically observed base pairs. By this account, an alphabet involving  $\kappa-X$  and  $\alpha-\Gamma$  base pairs would ensure equally faithful replication as the conventional alphabet. In vitro experiments for one of these base pairs suggest that they are indeed serious candidates for an alternative nucleotide alphabet (442). These experiments have been carried out with  $\pi$ , a methylated form of  $X$  that is easier to incorporate into oligonucleotides by nonenzymatic synthesis. Piccirilli and collaborators (442) synthesized oligodeoxinucleotides that contain  $\kappa-\pi$  pairs and found that their stability is comparable to oligodeoxynucleotides involving the conventional four nucleotide building blocks. Moreover, base mispairing involving  $\pi$  and  $\kappa$  destabilizes double helices to an extent



**Figure 2.3** The number of complementary hydrogen donor–acceptor groups for different combinations of chemically stable bases. Light gray fields correspond to chemically unstable bases or their complements. Black fields correspond to base pairs with the maximum number of complementary donor–acceptor groups. (a) The three digits next to each base indicate the hydrogen donor–acceptor pattern, as explained in the text. (b) Similar to (a), except that the rightmost digit next to each base indicates whether the base is a purine (0) or pyrimidine (1). The matrix of base pairs is organized such that even parity, odd parity, and mixed parity bases are grouped together in blocks. Names of nonstandard bases ( $\Gamma$ , X,  $\beta$ ,  $\kappa$ ,  $\alpha$ , aA) are taken from (442). The chemical structure of four nonstandard bases is shown in Figure 2.2. aA stands for amino adenine, the adenine analogue that differs from adenine by having an additional amino group at the lowermost base pairing position. It is thus able to have three hydrogen bonds with the complementary base U, whereas adenine can have only two such bonds.

comparable to that for mispairing involving the four conventional bases. In addition, DNA and RNA polymerases readily incorporate nucleotides carrying the base X opposite to  $\kappa$  with good fidelity.

### Genetic Alphabets and Error-Resistant Codes

MacDónaill also points to a curious analogy between the base alphabet and parity codes in computer science. This analogy adds another facet to the above argument, and it may help answer why we do not see additional nucleotides in genetic material. In computer science, a parity code is a representation of binary information that permits effective error detection in information processing. In a parity code, the total number of bits in a block of information, such as a byte consisting of eight bits, must add up to an even number (in an even-parity code) or to an odd number (in an odd-parity code). If one of the bits has been accidentally changed in value, parity is compromised, which allows detection of the error. In a parity code, one or more bits (the parity bits) are devoted to ensure that the sum of other bits adds up to an odd or even number. MacDónaill realized that one can represent bases and their analogues as four-letter binary words if one adds to the above representation of donor-acceptor configurations one bit that corresponds to the chemical nature of the base. Specifically, one can define a zero as corresponding to a purine, and a one as corresponding to a pyrimidine. (This choice is arbitrary and could be reversed without affecting the following argument.) In this representation, G becomes 0110 (as opposed to just 011), and U becomes 0101. It then becomes evident that the realized alphabet of bases belongs to an even-parity code. That is, all conventional bases have even parity (Figure 2.3b). The alternative (but not realized) optimal alphabet ( $\kappa$ -X,  $\alpha$ - $\Gamma$ ) would belong to an odd-parity code, because all its members have odd parity. The odd- and even-parity codes correspond to the upper and lower diagonal blocks in the scheme of Figure 2.3b. The upper-left and lower-right blocks correspond to mixed-parity codes. That is, base pairs in these blocks do not have the same parity.

This parity-code structure of nucleotides may well be accidental, but it allows a very compact representation of optimal nucleotide alphabets. As Figure 2.3b shows, alphabets where all nucleotides have either odd or even parity are maximally robust, in the sense that they discriminate best between complementary and noncomplementary nucleotides, and thus allow for most faithful replication of genetic material. The parity representation also allows a better understanding of the poor discrimination between noncomplementary bases in two of the blocks of Figure 2.3b. In alphabets of mixed parity, the distance between any two purine or any

two pyrimidine words can be as little as one. That is, any two bases with the same backbone (purine or pyrimidine) might differ at as little as one of their hydrogen donor–acceptor patterns. They are thus chemically very similar in their ability to pair with other bases. For example, the distance between the even-parity C (1001) and the odd-parity  $\kappa$  (1011) is only one.  $\kappa$  is complementary to X, also an odd-parity base. The  $\kappa$ -X base pair is strongly binding, because hydrogen bonding can occur at all three possible hydrogen bonding positions. However, because C is so similar to  $\kappa$ , it can also pair effectively with X, having two out of three complementary opposite hydrogen donor–acceptor configurations. This makes for poor discrimination between the complementary pair  $\kappa$ -X and the noncomplementary C-X. In an alphabet of only odd-parity or even-parity nucleotides, this similarity of bases with the same backbone is avoided. Parity enforces that any two purine and any two pyrimidine bases differ in at least two of their hydrogen donor–acceptor configurations.

## Caveats

Again, the representation of Figure 2.3 merely counts matching hydrogen donor–acceptor patterns at juxtaposed bases. It is thus but a caricature of base-pairing chemistry. Reassuringly, more detailed quantum chemical calculations of binding energies support the qualitative, crude picture of Figure 2.3 (352). However, such calculations do not account for additional factors influencing base-pairing fidelity. For example, hydrogen donor–acceptor groups within a given base pair may be aligned in different ways, most notably through wobble pairing. Wobble pairing is due to the bases' freedom to move slightly within DNA. To address how alternative pairing configurations may influence binding energy, Száthmary (535) calculated base binding energies for base analogues listed in Figure 2.3 and for several alternative pairing configurations, including wobble, from published binding energies of functional groups in the bases. Significantly, he found that a simple hydrogen donor–acceptor matching pattern such as that shown in Figure 2.3 may lead to the same qualitative conclusion as the more complex calculation (536). Thirdly, base pairing is not all there is to the integrity of genetic material. For instance, interactions of base pairs within a double helix are also important. In such interactions, steric differences between bases, as well as energetic contributions of stacked bases in the helix, need to be considered (144, 300, 379, 380). Finally, replication enzymes may prefer some bases to others when reproducing genetic material. The importance of this factor is most difficult to evaluate: Early replication enzymes may have had substantially

different base preferences than present-day enzymes, partly because they were RNA-based. In addition, replication enzymes may have coevolved with the genetic alphabet. Thus, studying current replication enzymes may not answer how such enzymes have influenced the evolution of the genetic alphabet.

### Is the Genetic Alphabet a Historical Accident?

If the basic hydrogen donor–acceptor scheme outlined in Figure 2.3 captures some of the chemistry important for base discrimination, the above question—why do we not see the alternatively optimal base pairs  $\kappa$ -X or  $\alpha$ - $\Gamma$ ?—has been partially answered. We may not see  $\kappa$ -X or  $\alpha$ - $\Gamma$  *in addition* to the conventional nucleotides, because the resulting alphabet might show poor discrimination between complementary and noncomplementary nucleotides when all possible combinations are considered. In the parity analogy, having these nucleotides in addition to the conventional ones would imply a mixed-parity code with little robustness against replication errors. But this analysis does not explain why the alphabet of DNA and RNA does not contain *exclusively* the alternative bases. Two principal possibilities exist. First, our present alphabet may be the result of a historical accident. Had life's tape been run twice, to say it with Stephen Jay Gould (203), we might well have the  $\kappa$ -X and  $\alpha$ - $\Gamma$  alphabet—leaving us to wonder why we do not have the A-U and G-C alphabet. However, a second possibility is that hidden chemical constraints have prohibited this alphabet from evolving. Orgel, for instance, has speculated that the synthesis of the conventional bases was more likely than that of the alternative bases under prebiotic conditions (417). In a similar vein, early replication enzymes may have replicated the conventional bases more speedily and accurately, or some of the alternative bases may have instabilities that are not obvious. For example, theoretical calculations suggest that the nucleotide  $\alpha$  may coexist with a tautomer that might allow  $\alpha$  to pair with bases other than its complement  $\Gamma$  (353).

In sum, the existing nucleotide alphabet may provide optimal discrimination between complementary and noncomplementary base pairs. Such discrimination endows DNA and RNA sequences with optimal robustness to replication errors. However, an alternative alphabet of stable nucleotides with similarly good discrimination also exists. MacDónaill (351) argues that a mixed alphabet consisting of both conventional and alternative nucleotides shows poor robustness. To validate this hypothesis is difficult with currently available experimental evidence. However, if

true, the two alternative possible base alphabets cannot be reached from one another in evolution, because mixed alphabets are not robust to replication errors. Put differently, once an alphabet is established, it is locked in. The reasons why precisely our alphabet is locked in are unclear. They might involve historical accidents, chemical constraints in the early evolution of life, or differences between the standard and alternative bases.

# 3

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## The Genetic Code

What is robust? The physicochemical properties of amino acids in a protein and, ultimately, protein function.

What is it robust to? Point mutations in codons.

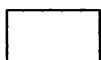
The genetic code is responsible for translating a sequence of nucleotide triplets (codons) into a protein's amino acid sequence. The vast majority of extant organisms use the code shown in Figure 3.1. Ever since this “universal” genetic code was discovered, the question of why this code and not another has received much attention. This question becomes especially significant if one considers how many possible genetic codes there are. That is, how many ways are there to encode 20 amino acids with 64 nucleotide triplets? The number of possible genetic codes is astronomical, even if one considers only codes that preserve the redundancy pattern of the universal code. The universal code has 3 amino acids to which 6 codons are assigned, eight amino acids with four codons, two amino acids with three codons, and nine amino acids with two codons. There are more than  $10^{65}$  possible genetic codes with this same distribution of  $x$ -fold degenerate amino acids. Why did nature pick the one we see? Here is part of the answer: The universal genetic code is more robust to changes in codon nucleotides than most alternative codes. Specifically, in the universal code, nucleotide changes are less likely to cause amino acid changes that affect the function of a protein.

### Three Complementary Hypotheses about Code Evolution

A single chapter could not do justice to the literature around the question of why nature picked the universal genetic code. You can find more comprehensive treatments elsewhere (292, 295, 420), but for my purpose, it will be sufficient to note that most of this literature crystallizes around three major hypotheses.

The first hypothesis emphasizes the role of stereochemical matches between individual codons and amino acids (179, 437, 618). According to

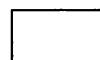
	<b>U</b>	<b>C</b>	<b>A</b>	<b>G</b>
<b>U</b>		UCU Ser	UAU Tyr	UGU Cys
		UCC Ser	UAC Tyr	UGC Cys
	UUA Leu	UCA Ser	UAA TER	UGA TER
	UUG Leu	UCG Ser	UAG TER	
<b>C</b>	CUU Leu	CCU Pro	CAU His	CGU Arg
	CUC Leu	CCC Pro	CAC His	CGC Arg
	CUA Leu	CCA Pro	CAA Gln	CGA Arg
	CUG Leu	CCG Pro	CAG Gln	CGG Arg
<b>A</b>	AUU Ile	ACU Thr	AAU Asn	AGU Ser
	AUC Ile	ACC Thr	AAC Asn	AGC Ser
	AUA Ile	ACA Thr	AAA Lys	AGA Arg
	AUG Met	ACG Thr	AAG Lys	AGG Arg
<b>G</b>	GUU Val	GCU Ala	GAU Asp	GGU Gly
	GUC Val	GCC Ala	GAC Asp	GGC Gly
	GUA Val	GCA Ala	GAA Glu	GGA Gly
	GUG Val	GCG Ala	GAG Glu	GGG Gly



Acidic



Amide



Hydroxyl containing



Alkyl



Aromatic



Sulfur containing



Alkyl



Basic



STOP

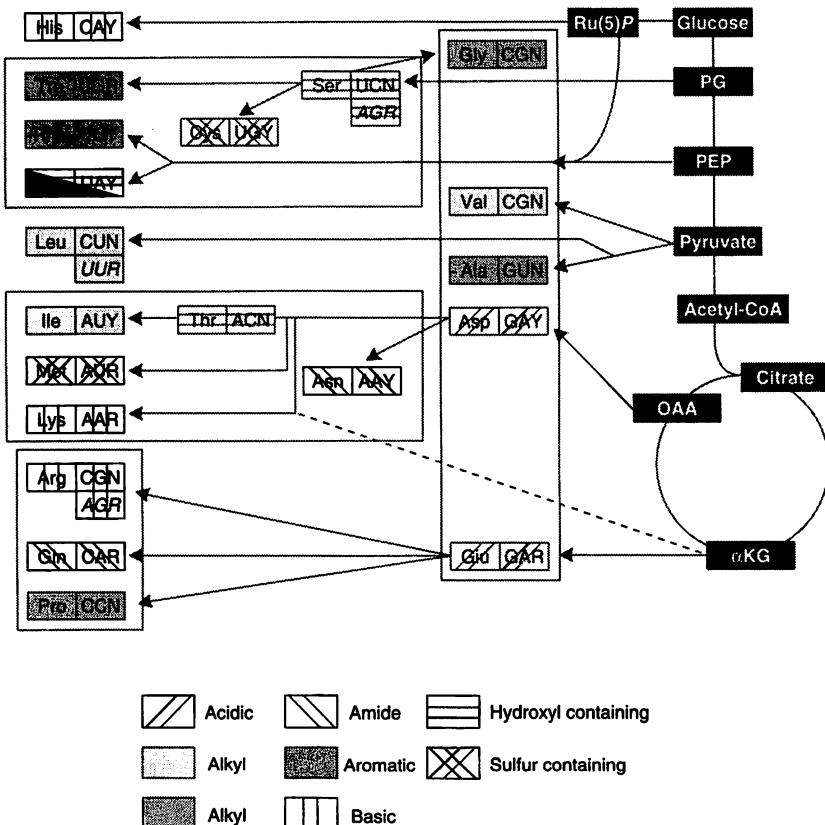
**Figure 3.1** The “universal” genetic code. Amino acids whose codons have U at the second position tend to be hydrophobic; those whose codons have A at the second position tend to be hydrophilic. Amino acids that share structural similarity tend to share codon sets connected by single point mutations. Ter, termination codon. From Figure 1 in (292).

this hypothesis, codon assignments originally resulted from chemical affinities between nucleotide triplets and individual amino acids. In the modern code, of course, there is no direct interaction between codons and amino acids. However, the argument goes, in a primitive translation machinery, today's highly specialized tRNA adaptor molecules and aminoacyl tRNA synthetases did not yet exist. Codons were matched to amino acids more directly, and amino acids with chemical affinity to particular nucleotide combinations would preferably become associated with these nucleotides in the code. Arguments in support of this hypothesis include correlations between chemical properties, such as hydrophobicity, of different amino acids and their codons' nucleotides. Various binding mechanisms between amino acids and their codons, anticodons, or other related nucleotide motifs have also been proposed (292). The most direct evidence for this hypothesis comes from *in vitro* selection of RNA molecules that bind to amino acids. For example, RNA molecules that are artificially selected to bind arginine contain disproportionately many CGN/AGR triplets (where N indicates any nucleotide and R indicates G or A). These high-affinity triplets correspond to arginine codons in the universal code (293, 294).

The second, "biosynthetic" hypothesis of code origin attempts to explain the code's structure through the history of metabolism. It departs from the reasonable assumption that early proteins consisted of fewer amino acids than extant proteins, and it postulates that the genetic code evolved alongside the amino acid repertoire of proteins (624). According to this hypothesis, the few early amino acids occupied a much larger number of codons than they do in today's code.

An important (and uncontroversial) prerequisite for this hypothesis is that amino acid biosynthesis abilities of cells increased over time. Many amino acids thought to be early amino acids are biochemical precursors of later amino acids. This indicates that amino acid biosynthesis pathways have expanded in a stepwise fashion, adding enzymatic reactions that produce additional amino acids. Concurrently, codons originally assigned to an early amino acid may have subsequently been reassigned to later amino acids derived from the early amino acid. In this sense, the genetic code coevolved with life's amino acid biosynthesis repertoire.

The main support for the biosynthetic hypothesis comes from the structure of the code itself. For instance glutamate (Glu) is a biochemical precursor of glutamine (Gln), and their respective codons (GAA/G for Glu and CAA/G for Gln) (Figure 3.1) are neighbors in the genetic code, i.e., they differ by only one nucleotide. Similarly, threonine (Thr) is a precursor of isoleucine, and their respective codons are also very similar. In addition, biosynthetic relations are reflected in the genetic code beyond



**Figure 3.2** Biosynthetic pathways and codon assignments in *Escherichia coli*. Bounded areas highlight codons that share the same first base identity.  $\alpha$ KG,  $\alpha$ -ketoglutarate; OAA, oxaloacetic acid; PEP, phosphoenolpyruvate; PG, phosphoglycerate; Ru(5) P, ribulose 5-phosphate. Data from (541). From Figure 3c in (292).

relations between neighboring codons. For example, amino acids derived from the same biosynthetic pathways tend to share the same first nucleotide in their codons (541). Figure 3.2 shows an overview of the relation between biosynthetic pathways of amino acids and codon assignments in *Escherichia coli* (292).

The evolution of aminoacyl-tRNA synthetases provides further tentative support for the biosynthetic hypothesis. Aminoacyl-tRNA synthetases are the enzymes responsible for attaching an amino acid to its transfer RNA (tRNA), the molecule carrying the amino acid to the ribosome

for protein biosynthesis. Some aminoacyl-tRNA synthetases for biosynthetically related amino acids shared a common ancestor more recently than aminoacyl-tRNA synthetases for unrelated amino acids (396). This suggests that the evolutionary origins of different aminoacyl-tRNA synthetases mirrored the evolution of amino acid biosynthesis pathways. Unfortunately, tRNAs themselves, whose evolutionary pattern would be of great interest in this regard, are unlikely to provide useful phylogenetic information. The reason is that they can easily switch allegiance between different amino acids (474), such that current tRNA codon assignments may not reflect the evolutionary history of tRNAs.

It is worth mentioning that even randomized genetic codes, codes where codon assignments have been shuffled, show similar codons for biosynthetically similar amino acid. Part of the reason is that numerous biochemical pathways can convert amino acids into each other (17, 18, 466). However, despite this caveat, the biosynthetic hypothesis can explain some aspects of the code difficult to explain otherwise. For example, some amino acids have very different chemical properties but similar biosyntheses and similar codons. This observation is better explained by the biosynthetic hypothesis than the stereochemical hypothesis, because the stereochemical hypothesis would predict dissimilar codons for chemically dissimilar amino acids.

These first two hypotheses about the genetic code's structure emphasize historical factors. The stereochemical hypothesis focuses on chemical affinities between nucleotides and early amino acids. The biosynthetic hypothesis emphasizes the history of amino acid biosynthesis. The remaining hypothesis emphasizes the code's robustness, and that this robustness arose during the code's evolution (140, 193, 513, 616). To be precise, it is not the code itself that is robust. What is robust is the chemical composition of amino acids in a protein. It is robust to point mutations in codons or to errors in translation. In other words, according to this hypothesis the code has evolved such that similar codons encode amino acids with similar physicochemical properties. Most mutations or translation errors will lead to substitutions of amino acids with similar chemistries, substitutions that change a protein's features minimally, and thus are unlikely to impede its function.

The historical and the robustness hypotheses are not mutually exclusive. For example, in the earliest stages of code evolution stereochemical factors may have been of paramount importance. Later, newly added amino acids and their need for codons may have supplanted stereochemistry as a driving force. Robustness could have evolved either during these two stages of code evolution or afterwards (292).

## Assessing Code Robustness Is Not Simple

At first sight, it seems straightforward to find out whether the genetic code is unusually robust. One can generate all possible alternative codes and measure to what extent neighboring codons encode chemically similar amino acids. However, this strategy contains several pitfalls. First of all, generating alternative genetic codes and evaluating their robustness to all possible point mutations requires fast computers. Thus, most early work arguing in favor of the robustness hypothesis relied on qualitative observations about the structure of the code (140, 193, 513, 616). To give but one example, most hydrophobic amino acids have a U at the second position of their codon, whereas most hydrophilic amino acids have an A. Unless a mutation interchanges these very nucleotides at the second codon position, the hydrophobicity of an amino acid—a key physicochemical property—will remain unchanged. It was not until electronic computers became widespread that such qualitative considerations could be left behind and the space of possible codes could be exploited more systematically. Even so, it is impossible to explore this space exhaustively, and one has to make choices about the biologically relevant parts of the space.

The second problem is this: What kind of amino acid substitutions have a small impact on a protein's function? Which amino acid properties should change minimally as a result of an “average” mutation or translation error? The most prominent among multiple-candidate properties are hydrophobicity, molecular volume, and isoelectric point. Among them, hydrophobicity has received the greatest scrutiny. Part of the reason is that interactions among hydrophobic amino acids are thought to hold the hydrophobic core of many proteins together. Any choice of one chemical property, however, is to some extent ad hoc, because some other property or a combination of properties may be more important. To circumvent this problem, one can consider only amino acid changes that have actually occurred during the evolution of related proteins. I now briefly outline how this can be done (173).

In general, three types of changes can occur in a protein's amino acid sequences: changes that are deleterious to the protein's function, neutral changes that do not affect the protein's function, and beneficial changes that are favored by natural selection. Deleterious changes are eliminated from the evolutionary record, because their bearers die. As a result, only neutral and beneficial changes are preserved. If one assumes that most amino acid changes preserved in proteins of related or identical functions are neutral, then one can use the observed frequency at which an amino acid X changed into amino acid Y as an indicator of the chemical similarity between X and Y. An X-Y exchange that occurred rarely

indicates that changing X into Y is likely to have a deleterious effect, and thus that X and Y are physicochemically dissimilar. Margaret Dayhoff compared the amino acid sequence of a wide spectrum of evolutionarily related and functionally similar proteins, and she determined how often one amino acid was exchanged for another for all 190 possible pairs of amino acids. She termed the resulting matrices of amino acid exchange frequencies PAM matrices (102). PAM stands for point accepted mutations but is often (incorrectly but more intuitively) interpreted as “percent accepted mutations.”

This approach has one important caveat. PAM matrices of distantly related proteins have a different structure from those of closely related proteins. Part of the reason is that in very closely related proteins, the vast majority of amino acid changes are due to single point mutations that transform one codon into another. However, in very distantly related proteins, any one codon may have suffered more than one mutation, and most observed amino acid changes will thus involve nonneighboring codons. This difference is important in assessing whether the genetic code is robust. Recall that one is looking for a measure of physicochemical similarity between amino acids. In distantly related proteins, the observed frequency of exchanges between two amino acids X and Y indicates their chemical similarity. However, in closely related proteins, exchanges that involve amino acids with nonneighboring codons will be exceedingly rare, simply because few mutations occurred and the majority of them transformed neighboring codons into one another. The PAM matrix of closely related proteins thus reflects the structure of the genetic code itself rather than chemical similarities among amino acids. Thus, one can use observed frequencies of amino acid exchanges in proteins of similar function as an indicator of amino acid similarity, as long as the proteins are only distantly related. This approach avoids having to choose indicators of physicochemical similarity ad hoc.

A third problem is how to compare robustness between the universal code and other codes. Two different approaches exist. Both start with the same basic procedure. One generates a genetic code at random and evaluates the average chemical similarity of amino acids—either via a physicochemical property or an observed frequency of amino acid exchange—encoded by all pairs of neighboring codons, that is, codons one nucleotide change apart. This average similarity  $\Delta$  is a measure of robustness for this code. By repeating this procedure for many randomly generated codes, one obtains a distribution of robustness. From here on, the two approaches diverge. One of them uses the following quantity:

$$\frac{\Delta_{\text{mean}} - \Delta_{\text{code}}}{\Delta_{\text{mean}} - \Delta_{\text{opt}}} \times 100 \quad (3.1)$$

Here  $\Delta_{\text{mean}}$  is the average robustness of all randomly generated codes,  $\Delta_{\text{opt}}$  is the code with the maximally possible robustness, and  $\Delta_{\text{code}}$  is the universal genetic code (114, 115, 194, 625). Expression 3.1 measures the robustness of the universal code as a percentage of the maximally achievable robustness, which is expressed as the difference in robustness between the average random code and the maximally robust code  $\Delta_{\text{mean}} - \Delta_{\text{opt}}$ . The other procedure does not use the mean robustness of all randomly generated codes but examines its distribution and asks, what fraction of randomly generated codes is more robust than the universal code (171, 173, 219)? The two procedures can give vastly different answers, which are the source of most conflicting results in the literature on code robustness. Here is the reason for this difference. The distribution of robustness among randomly generated codes is approximately normal (173). For the purpose of illustration, if the maximally robust code is 10 standard deviations more robust than the average random code, and if the universal code is 5 standard deviations more robust than the average, the universal code shows only 50% of the maximally achievable robustness. However, at the same time, fewer than one in a million codes are more robust than a code that is 5 standard deviations above the mean.

Although the choice of procedures is to some extent a matter of taste, it may be biologically more sensible to determine the fraction of codes more robust than the universal code. The reason is that the maximally robust codes may occupy a vanishingly small fraction of the space of all possible codes. Given that several historical factors constrain the evolution of the code, a maximally robust code may not even have been attainable in evolution (174).

A final problem in evaluating code robustness is that not all point mutations are equally likely. That is, for any one codon, not all mutational transitions to neighboring codons are equally probable. The most conspicuous deviation from equiprobability is known as the transition:transversion bias. Transition mutations, mutations that change pyrimidine bases into pyrimidine bases, and purine bases into purine bases ( $A \leftrightarrow G, C \leftrightarrow U$ ), are generally more frequent than transversion mutations ( $A, G \leftrightarrow C, U$ ) (82, 316, 336, 386, 388). The reasons lie in nucleotide chemistry, suggesting that a similar bias may also have occurred earlier in life's evolution, when the genetic code was most plastic. Only some of the work on code robustness takes this bias into account.

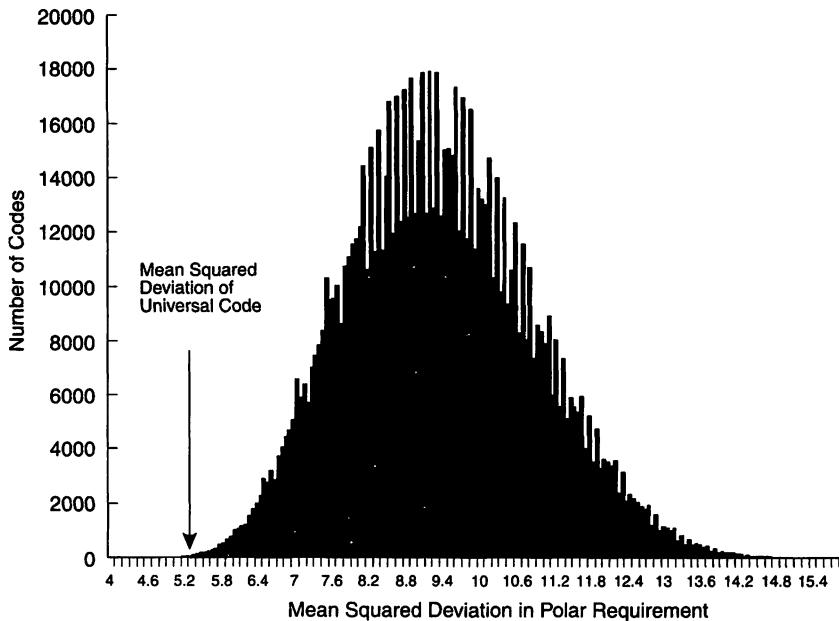
### The Case for Code Robustness

Haig and Hurst (219) generated random codes with the same code degeneracy as the universal code (3 amino acids with 6 codons, 8 amino acids

with 4 codons, etc.) They did so by leaving the codon blocks encoding the same amino acids (Figure 3.1) intact, but reshuffled all 20 amino acids among these blocks. There are  $20! > 10^{18}$  possible ways to do that, so the codes thus generated are only a subset of all possible codes. For each of  $10^6$  randomly generated codes, and four different measures of physicochemical amino acid properties, they determined by how much any two neighboring amino acids—amino acids whose codons differ in one nucleotide—differed in their physicochemical properties. Specifically, they examined each neighboring amino acid pair, calculated the mean-squared difference in a physicochemical property for this pair, and averaged this mean-squared difference over all neighboring pairs. Codes with a lower mean-squared difference are more robust, because the average amino acid substitution changes physicochemical properties less.

By this criterion, only one in 10,000 randomly generated codes turned out to be more robust than the universal code with respect to the polar requirement of amino acids (219), an indicator of hydrophobicity (617). Put differently, under the universal code, point mutations would perturb the hydrophobicity of amino acids less than in 99.99% of randomly generated codes (Figure 3.3). This assessment of code robustness had not yet taken transition:transversion errors into account. To do that, Freeland and Hurst weighted different neighboring codons differently, according to whether they differed by a transition or a transversion mutation (171). For example, the mutation UUU → UCU (Phe → Ser) involves a transversion and would thus be less frequent than the mutation UUU → UAU (Phe → Tyr), a transition. Robustness of the universal code with respect to hydrophobicity increases steadily as the transition:transversion bias is increased from one until it reaches a maximum at a bias of three. At this maximum, the universal code is an additional twofold more robust than random codes: One in 20,000 randomly generated codes is more robust than the universal code. Code robustness then decreases slightly as the bias is increased further. In extant species, transitions are more frequent than transversions by a factor 1.7–5, depending on species and genes studied (82, 316, 336, 386, 388). Thus, maximal robustness is reached at a transition:transversion bias that lies within the empirically observed range.

While code robustness is striking with respect to hydrophobicity, it is much less pronounced for other physicochemical properties. For example, some 30% of randomly generated codes preserve the molecular volume of amino acids more faithfully than the universal code, and some 90% are more robust with respect to the isoelectric point of amino acids (219). Without a priori knowledge that hydrophobicity is relevant for robustness of protein function and other quantities are not, one cannot exclude the possibility that code robustness to hydrophobicity changes is



**Figure 3.3** Histogram for the mean-squared deviation in polar requirement of amino acids encoded by neighboring codons, as obtained from 1 million randomly generated variants of the universal genetic code. Polar requirement is an indicator of hydrophobicity (617) and a low mean-squared deviation is an indicator of high code robustness. The horizontal axis shows this indicator of robustness. The vertical axis shows the number of random variant codes generated with the mean-squared deviation shown on the horizontal axis (from a sample of one million random codes). The arrow shows the robustness of the universal code: The mass of the curve to the right of it indicates the number of less robust codes among the random variants. There are only 114 more robust variants, i.e., a fraction  $p = 0.000114$  of random variants are more robust than the universal code. Modified from Figure 1a of (171).

an artifact of this choice. For example, according to the stereochemical hypothesis, the code structure is influenced by chemical affinities between nucleotides and amino acids. Two amino acids with similar hydrophobicities, as measured by their polarity requirement (617), may also bind to similar nucleotides and thus share similar codons. So, a code whose evolution has been influenced by stereochemistry might artifactually appear robust to changes in hydrophobicity.

To address this problem Freeland and collaborators (173) used a measure of amino acid similarity independent of chemistry, namely the preserved changes of amino acids in distantly related proteins according to

Dayhoff's PAM matrices (102). As I discussed above, frequently found exchanges of an amino acid X for an amino acid Y indicate chemical similarity between X and Y. When codes are scored according to their robustness with respect to this measure, the results are even more striking than for hydrophobicity. Among one million genetic codes generated at random as described above, not one is more robust than the universal code under realistic values for the transition:transversion bias (173).

All observations I have surveyed so far were generated under one specific randomization scheme that preserves the redundancy patterns observed in the universal code. Would less stringent randomization lead to different results? Probably not. In a large sample of codes where codons are assigned at random to different amino acids, thus destroying the redundancy patterns of the universal code, the resulting average robustness is at least 20% lower than the average robustness of random codes generated through the more conservative amino acid swapping scheme. This means that the universal code's robustness would stand out even more starkly against more completely randomized codes (173). (Judson and Haydon carried out a comprehensive analysis of code robustness using (3.1) and various physicochemical criteria under complete randomization. (265))

A final question regards the biosynthetic similarity of amino acids that are neighbors in the code and how this similarity relates to code robustness. Many biosynthetically similar amino acids have similar physicochemical properties. If neighboring amino acids in the code are biosynthetically (and thus often physicochemically) similar, apparent code robustness may simply be an artifact of the code's history, of its coevolution with amino acid biosynthesis. To address this question, one can randomize the universal code such that all reshuffled codes preserve similar codons for biosynthetically related amino acids. To assess robustness under this scenario amounts to separating the influence of code history and selection on robustness. The simplest way to restrict code reshuffling to related amino acids takes advantage of the fact that many biosynthetically related amino acids share the same first nucleotide. One can thus allow codon assignments to change only among (but not within) groups of amino acids whose codons share the same first nucleotide. With this restriction, there are  $2 \times 10^9$  possible random codes. The universal code stands out dramatically among them. When hydrophobicity is used to assess robustness, the universal code is more robust than 99.97% of randomized codes (172). When evolutionarily accepted amino acid pairs are used as a criterion, no more than five out of one million randomly generated codes fare better than the universal code (173). This subset of randomized codes also achieves nearly the maximally possible robustness (>96%) according to the more stringent criterion in (3.1). Thus, if one

takes into account the possible historical constraints of amino acid biosynthesis relationships, the universal code is nearly maximally robust.

### Point Mutations or Translation Errors?

The first genetic code evolved most likely in organisms with an RNA genome. Judging from modern genetic systems such as RNA viruses, mutation rates in an RNA world may have been orders of magnitude higher than in a DNA world. Thus, selection to reduce the deleterious effects of mutation may have been an important factor in the evolution of code robustness. On the other hand, even though mutations may have been very frequent, translation errors may have been an equally or more important source of amino acid changes, especially because any one RNA molecule may be translated many times. Woese argued that the earliest translation apparatus may have been so error-prone that early proteins are best thought of as “statistical” proteins (616). That is, only rarely would a gene’s codons be translated twice in exactly the same way. The protein products of a gene would thus show wide variation in biological activity. It would have been very difficult to evolve the sophisticated machinery of modern translation—each of whose parts has highly specific tasks—under these conditions, simply because doing so would require accurate translation in the first place. One way to alleviate this problem is to evolve maximal robustness of protein function to translation errors, such that neighboring codons encode physicochemically similar amino acids. Thus, both point mutations and translation errors may have influenced the robustness of the genetic code. (Chapter 17 gives a more general perspective on the relation between genetic and nongenetic errors in the evolution of robustness.)

Does the structure of the code itself bear traces of the selection pressures that may have acted on it? That is, does the code have features that would be expected only if either point mutations or translation errors had been the driving factor behind the evolution of code robustness? Freeland and Hurst provide evidence that selection for translational errors may have left traces in the code’s structure (171).

Translation errors do not occur equally frequently across different nucleotides in a codon. Specifically, the error rate is often much greater at the third position (III) of a codon than at the first position (I), whose error rate is in turn greater than that at the second position (II) (176, 432, 616). Schematically,

$$\text{III} > \text{I} > \text{II}$$

For example, in the *in vitro* translation of mRNA that contains only U residues, mRNA whose codons all encode phenylalanine, position III

shows a 100-fold higher error rate than position I, whose error rate is 10-fold higher than that of position II (616). In addition, codon positions differ in the frequency of transitional versus transversional mistranslation errors (176, 432, 616). Both *in vivo* and *in vitro* translation errors increase in suboptimal chemical environments (high pH, low temperatures, low magnesium level, exposure to antibiotics, etc.). Most such conditions cause the same types of translation errors, suggesting that the type of error is determined by the chemistry of translation itself rather than by any one physiological condition (616). Note that in contrast to translation errors, one would not necessarily expect systematic differences across codon positions for mutation rates. That is, there is no reason to assume that the second position in a codon would always be more or less mutable than the third or first position. This difference distinguishes the effect of mutations and translational errors on the genetic code.

Because translation errors occur at vastly different frequencies among codon positions, selection for robustness may have acted to different extents on the different positions. That is, for position III, where most errors occur, selection for robustness would be the strongest, whereas for position II it would be weakest. One might thus expect that different codon positions show different degrees of robustness. That is, neighboring codons that differ at their third nucleotides might encode more similar amino acids than neighboring codons that differ at their second nucleotides. Freeland and Hurst (171) found indeed that the degree of robustness at each of the codon positions mirrored exactly the observed pattern of translational errors. Schematically,

Translation error rates:	III > I > II
Robustness:	III > I > II

For example, while codon position III of the universal code is more robust than 99.98% of all randomly generated codes (with respect to hydrophobicity), codon position II is more robust than only 9% of randomly generated codes.

A complementary line of inquiry asks whether the genetic code is more robust than randomly chosen codes if one includes mistranslation error rates in codon–neighborhood relations. For example, amino acids encoded by codons that differ at position III (where the translation error rates are high) are much more likely to be substituted for each other than amino acids whose codons differ at position II. Code robustness under this error pattern—typical for mistranslation—is 100-fold higher than under the uniform error pattern typical for mutation. (171). While these findings do not exclude a role for mutations in the evolution of code robustness, they do indicate an important role for translation errors.

## Present-Day Code Evolution

The “universal” code from Figure 3.1 is by no means universal. By now, several dozen variant codes are known both in nuclear and mitochondrial genomes of a variety of organisms (295). At first sight, it is difficult to understand how such variants can evolve, because reassigning only one codon to a new amino acid would have a drastic effect on most proteins in a cell. However, several proposals have been put forth to explain continuing code evolution (295). The observation of variant codes invites the question of whether evolutionary improvement of code robustness continues to this day. At first sight, this would seem unlikely, because the strong selection pressures that existed in early genomes—through high mutation rates or low translation fidelity—do not persist to this day. And, indeed, modern code variants are no more robust than the universal code. Most of them are, in fact, slightly less robust (173). The code’s evolution toward increased robustness may thus have ceased in the distant past.

In sum, the present-day genetic code is more robust to mutations and translation errors than the vast majority of its randomly chosen variants. This holds under a variety of biologically sensible regimes of code randomization, including one that preserves similar codon assignments for amino acids with similar biosyntheses. It also holds regardless of whether hydrophobicity or an evolutionary indicator is used to gauge similarity among amino acids. In addition, it holds under realistic assumptions about how mutations change nucleotides, especially the well-known transition to transversion bias. We do not know whether natural selection acted on robustness to mutations, to translation errors, or both. However, the different extent of robustness among the three codon positions suggests that selection to minimize translation error played at least some role in the evolution of code robustness.

# 4

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## RNA Structure

What is robust? An RNA molecule's secondary structure.

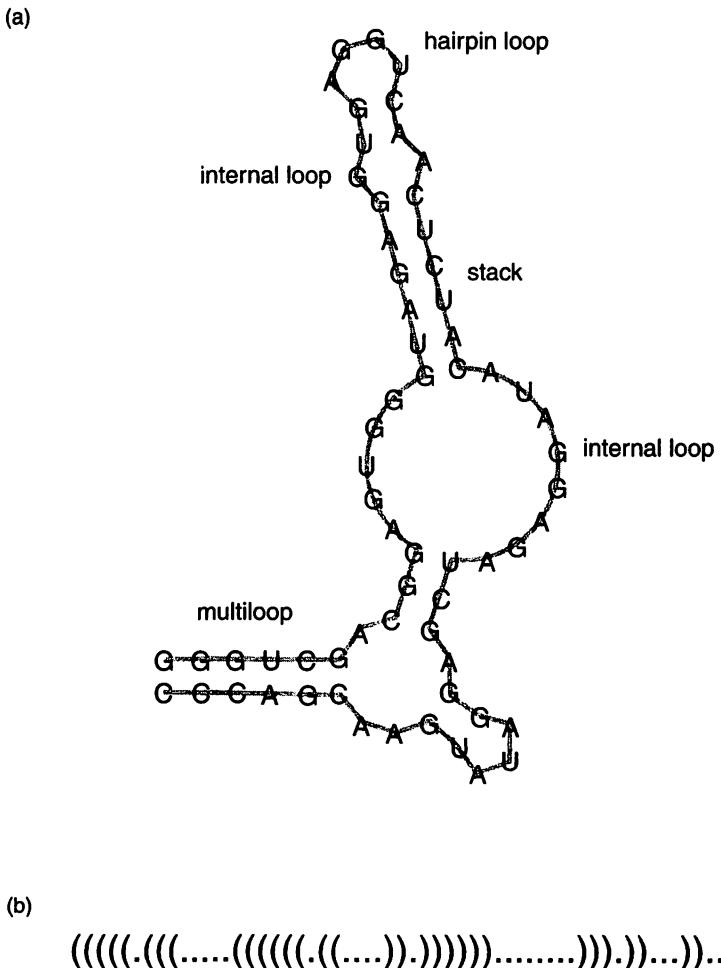
What is it robust to? Changes of individual RNA nucleotides.

The currently most explicit and deepest analyses of mutational robustness regard the structure of RNA. These analyses not only show that RNA structures can be very robust to changes in individual RNA nucleotides, they also characterize the vast neutral space associated with many RNA structures and show how RNA robustness can evolve within this neutral space; they demonstrate that neutral mutations can be critical to evolutionary innovation in RNA structure; and they point to a link between mutational robustness and robustness to nongenetic change.

RNA is an attractive molecule for studies of mutational robustness, because of its importance in past and present life. It may have been life's earliest information carrier and biocatalyst. And although DNA and proteins have superseded RNA in these roles, RNA still is involved in most of life's key processes. Examples go well beyond the well-known messenger, transfer, and ribosomal RNAs. They include small nuclear RNAs, which are key parts of the splicing machinery; guide RNAs important in RNA editing; telomerase RNA necessary for maintaining chromosome ends; the signal recognition particle RNA involved in translocating proteins through membranes; and RNA molecules regulating gene expression through RNA interference.

### Structure and Function of RNA

To understand the function of most RNA molecules, one must know not only their sequence of A, U, C, and G nucleotides, but also their secondary and tertiary structures. RNA secondary structure is an elaborate planar shape that is formed when an RNA molecule folds onto itself, thus forming hydrogen bonds of complementary base pairs within the molecule (between A-U, G-C, and, to a lesser extent, G-U nucleotides) (Figure 4.1).



**Figure 4.1** Two equivalent representations of RNA secondary structure. (a) Typical two-dimensional graphical representation. Stacks, regions of paired bases, and various kinds of loops, unpaired regions, are indicated. Base pairs in an RNA secondary structure have to meet two conditions: First, each nucleotide must be paired with at most one other nucleotide. Second, two pairings cannot cross in the planar projections of the structure, otherwise planarity would be violated. (b) Dot-parenthesis representation. A dot stands for an unpaired base, and a pair of matching parentheses corresponds to a base pair. The two representations are equivalent.

The three-dimensional tertiary structure brings distant secondary structure elements into proximity through nonstandard base pairing, pseudoknots, and bivalent ions such as Mg<sup>2+</sup>.

To fully understand the biological activity of RNA molecules, especially RNAs with catalytic activity, one needs to understand their tertiary structure. However, this structure is difficult to characterize (121). Compared to the number of solved protein structures, which number in the thousands, the number of well-characterized RNA tertiary structures is puny. Fortunately, an RNA's secondary structure is easier to determine, and it can provide important clues to an RNA's function. Take the example of group I introns, RNA molecules that catalyze their own excision from a larger molecule. Here, conserved parts of the intron's secondary structure reveal the location of its catalytic site necessary for excision (121).

The right secondary structure is critical to the function of many RNA molecules. Examples include secondary structures of many viruses whose genome consists of RNA, such as the TAR sequence and the Rev-1 responsive elements of human HIV, the internal ribosomal entry site of picorna viruses, and the 3' untranslated region of flavivirus genomes (35, 103, 254, 356, 446). Their RNA secondary structures interact with parts of the protein machinery necessary to complete the viral life cycle. Prominent other examples include messenger RNA and the importance of its secondary structure for its maturation and for the efficiency of its translation into protein (357, 411, 436, 563). The importance of RNA secondary structures is further underscored by multiple evolutionary studies. Such studies demonstrate that distantly related species harbor RNAs with diverged nucleotide sequences but conserved secondary structures. This indicates that natural selection maintains these secondary structures. Examples of evolutionarily conserved secondary structures come from ribosomal RNAs, transfer RNAs, catalytic RNAs such as ribonuclease P, and the 3' untranslated regions of some messenger RNAs. In addition to being conserved, such secondary structures often show evidence of compensatory mutations, which further indicates the importance of a structure's integrity. In an incidence of compensatory mutation, one species may contain a G-C base pair in a base stack (double-stranded helix), whereas this base pair may have been replaced by a A-U pair in another species, or vice versa. This requires two mutations (e.g., G → A and C → U), one of which compensated the other's effect on the secondary structure (168, 256, 257, 393, 426, 434, 619). In sum, both functional and evolutionary studies show that secondary structure, while not sufficient, is often essential for RNA function. Its formation and robustness thus deserve analysis in their own right.

## Analysis of Secondary Structure

Multiple experimental techniques to analyze RNA secondary structure are available (291, 378), and they have yielded many experimentally validated secondary structures. However, these techniques are generally too laborious to analyze the many variants of an RNA sequence necessary to understand its robustness against mutations.

Currently, the only alternative is computational determination of RNA secondary structures. The techniques for such predictions fall into two categories. The first uses RNA sequences from multiple different organisms to predict secondary structures. It relies on compensatory mutations that have occurred in these organisms (168, 256, 257, 393, 426, 434, 619). This approach assumes that secondary structure is conserved across the species examined. However, to fully understand a structure's robustness one needs to be able to ask how the structure varies in response to mutations. For this reason, this approach is less than ideal to understand RNA robustness. In addition, the approach works best if one has RNA sequence information from many species, a requirement met by intensely studied RNA molecules, such as ribosomal RNAs or some viral RNAs, but not by many other RNAs of interest. This leaves a second computational approach, the prediction of RNA secondary structure on the basis of thermodynamic principles. Such predictions can be made on several levels of resolution. First, one can determine the secondary structure that has the smallest free energy and is the most stable secondary structure. The task of predicting this minimum free energy structure is simplified by the fact that each secondary structure consists of only two kinds of elements: loops, that is, regions of unpaired bases, and stacks, regions of paired bases (Figure 4.1). Loops destabilize a secondary structure, whereas stacks stabilize it.

The most stable secondary structure is generally not the structure with the largest number of paired bases. Part of the reason is that each stack, although it stabilizes secondary structure, by necessity creates a loop, which destabilizes secondary structure. For instance, the transfer RNA responsible for attaching histidine to a nascent polypeptide has more than  $10^5$  secondary structures with 26 base pairs, the maximum number of base pairs possible for this RNA. However, it has only one minimum free energy structure, which has only 22 base pairs, fewer than the maximum number of 26 base pairs (634).

Most of a structure's stabilizing energy comes from interactions between the aromatic rings of adjacent pyrimidine and purine base pairs, so-called stacking interactions. Secondary structure prediction algorithms (405, 601, 646, 647) take advantage of experimentally determined energy

contributions of stacks and loops (226, 255, 360, 559, 594). Albeit not perfect, their predictions are generally in good agreement with experimentally determined secondary structures.

The next higher level of resolution in predicting RNA structure takes into account that each possible secondary structure an RNA can adopt, including the minimum free energy structure, is only metastable. That is, thermal fluctuations cause an RNA molecule to unfold and refold constantly, and thus to assume different secondary structures. Specifically, the time an RNA molecule will spend in a secondary structure with free energy  $E_i$  is proportional to  $e^{-E_i/kT}/\sum_{E_j} e^{-E_j/kT}$ . Here,  $T$  is the temperature in kelvins, and  $k = 1.4 \times 10^{-23}$  JK<sup>-1</sup> is the Boltzmann constant. The lower a secondary structure's free energy compared to other structures, the more time the RNA molecule will spend in this structure. The secondary structure with the lowest free energy—the minimum free energy structure—is the one in which the molecule will spend the most time. At ambient temperatures, thermal energy fluctuations allow an RNA to wiggle between alternative structures that occur within an interval of a few  $kT$  above the minimum free energy structure.

Algorithms to calculate the free energies of secondary structures within an energy range above the minimum free energy exist (634), but they are computationally more demanding than those determining the minimum free energy structure. Most existing work has thus examined RNA robustness on the level of the minimum free energy structure.

The final, highest level of resolution also takes into account an RNA's folding kinetics—the temporal order in which base pairs form as an RNA molecule is synthesized. Folding kinetics is an important determinant of structure, especially for long RNA sequences (231, 382). Algorithms that take folding kinetics into account (157, 217, 252, 376) are computationally demanding and have not yet been used to study structural robustness.

## RNA Sequence and Structure Space

An RNA sequence composed of  $n$  nucleotides can be thought of as a point in a space of sequences. To be sure, this space is very different from any space we are familiar with. First, it has an enormous number of dimensions, one for each of the  $n$  nucleotides in the sequence. Second, each of these dimensions can assume only four discrete values, one for each of the nucleotides A, C, G, and U. The fact that RNAs occupy high-dimensional discrete spaces accounts for many of their counterintuitive features, which I now summarize (161, 490).

How are secondary structures distributed in RNA sequence space? This question can be asked on several levels of resolution. First, how

many sequences fold into any one structure, on average? The answer can be determined exhaustively for short sequences and estimated through combinatorial analysis for longer sequences (236, 490). The result is that the average number of sequences per structure is astronomical, even for sequences of moderate length. For instance, there are  $4^{20} = 1.10 \times 10^{12}$  RNA sequences with 20 nucleotides, but no more than 2741 distinct minimum free energy structures of 20 nucleotides. This implies that, on average, there are more than 400 million RNA sequences per structure, even for sequences as short as 20 nucleotides. The discrepancy between number of sequences and numbers of structures becomes much greater for longer sequences.

Such averages, however, are just as misleading as they are revealing. The reason is that not all structures are attained by an equal number of sequences. To the contrary, the number of sequences that fold into the same structure has a highly uneven distribution: Most structures are rare, that is, few sequences fold into them. Conversely, the vast majority of sequences fold into a small number of very frequent structures. For short sequences or sequences with a restricted alphabet of only two nucleotides, this distribution has again been calculated through exhaustive folding of all sequences in the space (214, 489, 490). For instance, there are more than  $10^9$  sequences of length  $n = 30$  that consist only of G and C nucleotides. Any one of these sequences folds into one of approximately  $2 \times 10^5$  structures, yielding on average 5000 sequences per structure. If one defines a frequent structure as one that is realized by more sequences than this average, then only 10.4% of structures are frequent. However, 93% of sequences fold into these frequent structures. At the other end of the structure spectrum, one finds 12,362 structures formed by only one sequence, and more than half of all possible structures are formed by fewer than 100 sequences. As a sequence's length increases, the frequent structures occupy an increasingly large fraction of sequence space. For very long sequences, almost all sequences fold into a vanishingly small fraction of structures.

What renders a minimum free energy structure frequent or rare? A balance between stacked and looped regions. While long stacks enhance the thermodynamic stability of a structure, they also reduce the number of sequences folding into the structure. The reason is that only complementary bases are allowed within a stack, which constrains the nucleotide sequences able to fold into that stack. Conversely, loops of unpaired bases can be formed by many different base combinations. That is, they are realized by a large part of sequence space. However, such loops are thermodynamically unfavorable and will not usually be prominent in minimum free energy structures. Frequent structures occupy the middle ground of high thermodynamic stability induced by stacks, balanced by

the realizability of loops. In such structures, paired bases are distributed among several stacks that are separated by loops. Fontana has pointed out that these frequent secondary structures may also be most suited to form complex and flexibly shaped tertiary structures, structures that are at the heart of an RNA's biological function (161). Put differently, biologically important structures may be frequent structures. Supporting this notion is the fact that blind evolutionary searches in sequence space will not be effective at finding rare structures in its vast expanses. Partly for these reasons, analyses of RNA robustness have focused on frequent structures.

## Neutral Neighborhoods

Two RNA sequences whose nucleotides differ at  $k$  positions are also called  $k$ -mutant or  $k$ -point neighbors of each other, because they can arise from each other by  $k$  changes of single nucleotides. A sequence that folds into a frequent structure typically has many one- and two-mutant neighbors that fold into the same structure. (The same holds for the neighbors of these neighbors.) Such neighbors are called neutral neighbors. As a consequence, one can hop via single nucleotide mutations from sequence to sequence, without ever leaving the set of sequences folding into the same minimum-free energy structure. Put differently, many sequence pairs in this set can be connected through a series of neutral mutations, mutations that leave secondary structure unchanged. This observation prompted Schuster and collaborators to call this set a neutral network (491). Evolutionary comparisons of biologically important RNA molecules underscore the biological relevance of this concept. Such comparisons, which identify the end points of an organismal lineage's explorations of sequence space, often show that vastly different sequences can adopt similar form and function. For example, only seven nucleotides are conserved among a prominent class of RNAs with catalytic activity, group I self-splicing introns. Nevertheless, secondary (and probably tertiary) structures within the core of these catalytic RNAs are conserved (338).

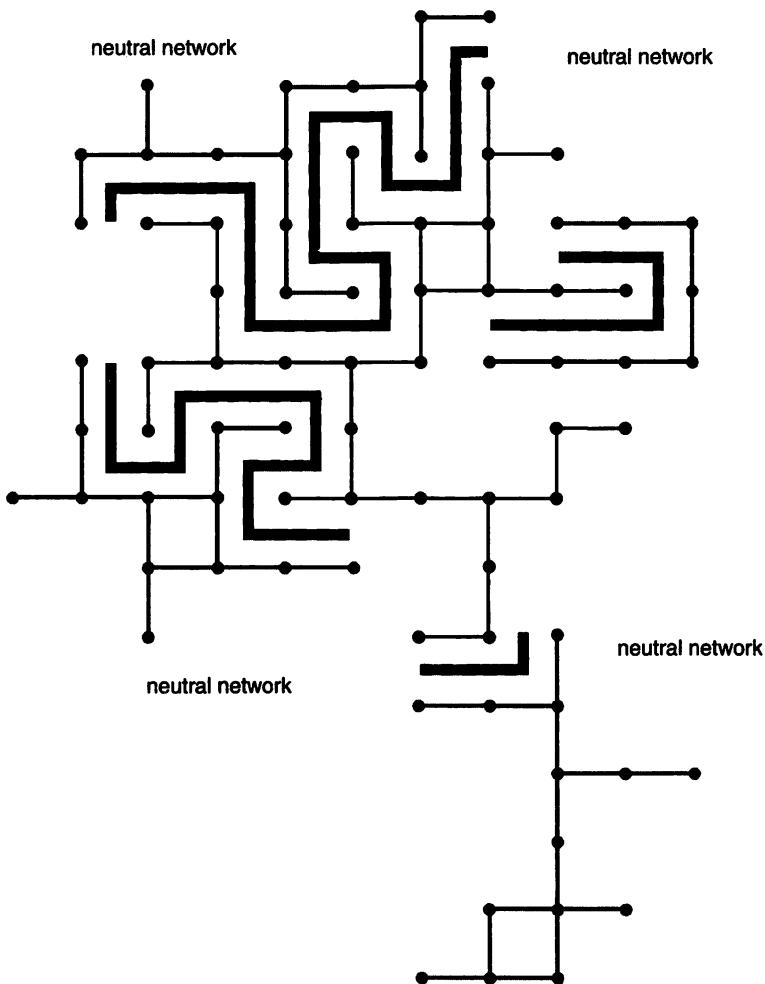
Mathematical analyses of sequence space indicate that the connectivity of the set of sequences folding into the same structure depends simply on the number of a sequence's neutral one-mutant neighbors (456, 457). (This set of sequences folding into the same structure is also called a structure's neutral set.) More specifically, if one chooses a sequence  $i$  at random from a structure's neutral set, some fraction  $\lambda_i$  of the sequence's one-mutant neighbors (among all possible one-mutant neighbors) will be neutral neighbors. If the mean fraction  $\lambda$  of neutral neighbors, i.e.,  $\lambda_i$  averaged

over all sequences  $i$  in the neutral set, exceeds the threshold  $1 - \kappa^{-1/(\kappa-1)}$ , most sequences in the neutral set are connected. Specifically, all sequences in the neutral set belong to one or a few equally sized subsets in this case. Any two sequences within each subset can be reached from each other through a series of single nucleotide changes.  $\kappa$  in the above formula is the number of different nucleotides that occur in the RNA molecule ( $\kappa = 4$  for the standard RNA alphabet AUCG).

If, on the other hand,  $\lambda$  does not exceed the above threshold, the neutral set can be subdivided into many subsets that are isolated from each other. These subsets have different sizes and some of them hold very few sequences (456, 457). Taken together, all this implies that the distribution of sequences folding into one structure has a simple relation to the structure's abundance: The more frequent a structure, the greater the average fraction  $\lambda$  of its sequences' neighbors that adopt the same structure, and the more likely it is that most of the sequences folding into the structure are linked through single point mutations.

A final, especially counterintuitive feature of RNA sequence space is that all frequent structures are near each other in sequence space. Consider a randomly chosen sequence that folds into a frequent structure. Now choose a completely different frequent structure and ask how far one has to step away from the original sequence to find a sequence that folds into this second structure. This question can be asked in more general terms: How large is the radius of the (high-dimensional) sphere around one sequence that is sufficient to find a representative of any common structure? Here, a sphere of radius  $r$  in sequence space is defined as a collection of sequences that differ in no more than  $r$  nucleotides from the sequence at the sphere's center. The worst-case scenario is that this radius is equivalent to the radius  $n/2$  of the entire sequence space. This means that one would have to traverse half of the sequence space to find a representative of every structure. However,  $r$ , which can be calculated (489–491), is much smaller than  $n/2$ . For instance, for RNAs of lengths  $n = 100$  nucleotides, a sphere of  $r = 15$  mutational steps contains with probability one a sequence for any common structure. This implies that one has to search only a vanishingly small fraction of sequence space (one  $4.52 \times 10^{37}$ th) to find all common structures.

In sum, sequence space is partitioned into a myriad neutral sets or networks, each of which contains all RNA sequences that fold into the same secondary structures (Figure 4.2). At the same time, a small sphere around any one sequence contains threads of all neutral networks that correspond to frequent structures. Thus, the neutral networks for different frequent structures are high-dimensional spider webs that interdigitate and penetrate each other, forming a filigree of filigree, a web of webs. If all this seems counterintuitive, it is only because we are reflexively



**Figure 4.2** Schematic illustration of interdigitating neutral networks for four secondary structures. (The structures themselves are not shown.) Each gray or black dot represents an RNA sequence. Connected dots correspond to one-mutant neighbors. Dots and edges of the same shading (gray or black) belong to the same neutral network. Thick lines with alternating shading indicate the shared boundary between two networks. After Figure 4 in (161).

drawing analogies between the only (three-dimensional) space we are familiar with and the high-dimensional discrete spaces of biological macromolecules. However, three-dimensional analogies are of little use in understanding high-dimensional spaces, and such spaces hold the key to understanding RNA's mutational robustness and its evolution.

## Studying Evolution in RNA Sequence Space

RNA was the subject of some of the earliest experiments studying evolution in the laboratory. In these experiments, natural selection *in vitro* generated shortened variants of the RNA genome of bacteriophage Q<sub>β</sub> that replicated very rapidly in the presence of the enzyme Q<sub>β</sub> replicase (516). Since then, various *in vitro* evolution approaches have successfully created RNAs with other desirable properties, such as specific catalytic activities or affinities to other molecules (137, 263, 318, 488, 558).

The rise of fast digital computers and RNA folding algorithms has also permitted recreation of RNA evolution in the computer. The computational work relevant to the analysis of RNA robustness (20, 163, 164, 248, 490, 491, 562) rests on a conceptually simple recreation of a flow reactor or chemostat in a computational environment. A flow reactor is a commonly used device in laboratory evolution experiments. It provides a continuous flow of nutrients needed by replicating objects—molecules here, whole organisms in other studies. A regulated outflow keeps the number of molecules in the reactor constant. Variation in these molecules is introduced through various protocols that generate random mutations. Under these conditions, rapidly replicating molecules that arise through mutation will accumulate and come to dominate the reactor over time.

The evolutionary processes that take place in such a flow reactor can be emulated computationally to study the robustness of RNA secondary structure (162, 191, 490). In this case, the molecules in the (emulated) flow reactor are RNAs. One can introduce new variation by changing, every generation, one randomly chosen nucleotide in a fraction  $\mu$  of molecules in the evolving population. A molecule's fitness—represented by its replication rate—is a function of its secondary structure. Biological motivations for relating replication rate to secondary structure are not hard to come by, considering that secondary structure motifs in many RNA viral genomes are important for viral replication (35, 103, 254, 356, 446). Thus, natural selection is emulated by allowing sequences to replicate more rapidly whose minimum free energy secondary structure is closest to a target structure. This approach requires a notion of distance between RNA secondary structures. There are various suitable distance measures, of which the most commonly used is the Hamming distance, which relies on the dot–parenthesis representation of structure (Figure 4.1): In calculating the Hamming distance between two structures, one aligns their dot–parenthesis representations and determines the number of positions at which these representations differ.

The pertinent computational work on RNA secondary structure evolution addresses two important questions. First, how does evolution occur

within a neutral network? This question regards the evolution of mutational robustness. Second, and even more elementary, starting with a randomly chosen RNA sequence, how does evolution arrive at a sequence folding into a desired structure?

### Structural Robustness and Its Evolution

I have thus far not explicitly discussed robustness of RNA structures against mutations. However, the mere fact that frequent RNA sequences form large connected networks with a common structure implies such robustness. It means that at least some of a sequence's immediate one-mutant neighbors have the same structure. However, my brief review on the structure of neutral networks omitted an important further observation. Neutral networks of frequent structures are not homogeneous. Any one such network consists of sequence islands where many sequences fold into the same structure, which are separated by regions that contain few sequences folding into the structure. In other words, not all sequences that are part of a neutral network have the same number of neighbors folding into the same structure. Some sequences have more such neutral neighbors, and are thus more robust to mutations, whereas others have fewer neighbors and are less robust (248, 562).

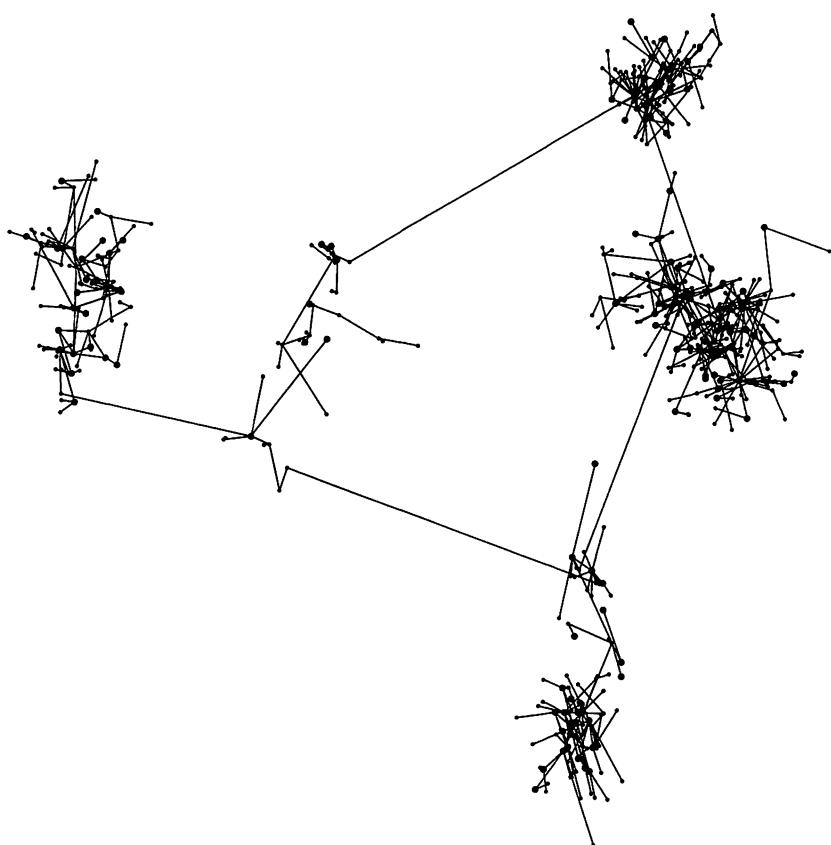
In an elegant mathematical and computational analysis, van Nimwegen and collaborators (562) showed what this structure of sequence space implies for the evolution of RNA molecules on a neutral network. I will now give a qualitative account of pertinent results (53, 562, 608) and defer a more quantitative treatment to chapter 16.

Imagine a large population of identical RNA molecules. Each of these molecules folds into the same frequent structure, a structure that is highly favored by natural selection. The entire population thus occupies one point on a vast neutral network in an even vaster sequence space. Individual members of the population suffer mutations that change one nucleotide with a probability  $\mu$  per generation. As a result of such mutations, some sequences fall off the neutral network, that is, they no longer assume the optimal secondary structure. Such mutant sequences quickly get eliminated from the population. Other mutations transform a sequence into another sequence on the neutral network, which has no consequences for the sequence's replication rate.

As time progresses, and more and more mutations occur, the population can be viewed as an expanding cloud of points that diffuses through this high-dimensional network. If the initial population started out in an island of high sequence connectivity—high robustness—its members will at first expand in sequence space to fill out this island. Then some of its

members will “discover” by random mutation the regions or “bridges” of low connectivity that connect the island to other islands. They will traverse these bridges, slowly, because on such a bridge many mutations will cause a sequence to fall off the neutral network and thus be eliminated from the population. Put differently, while on such a bridge, only a few (neutral) mutations will cause a sequence to advance one mutational step toward the next island. Once having traversed the bridge, a population’s members can spread through the new island and then go on to discover a third island, and so on. If the original population had started out on one of the low-connectivity bridges, it needs to first diffuse to the first island, from where its members can then discover other islands. Thus, over time, the population of RNA molecules explores an ever-increasing number of islands of high robustness. A snapshot of this process is shown in Figure 4.3. This image—just like my words—is at best a caricature of the evolutionary process, because it projects a high-dimensional sequence space onto two dimensions. The key insight, however, is independent from this geometrical analogy: Under selection to maintain a secondary structure, populations of evolving RNA molecules evolve increased robustness against mutations. They do so by accumulating in densely connected islands within a neutral network (53, 562, 608).

Is it possible to determine by how much robustness can increase in this process? The answer is yes (562). Large populations eventually reach an unchanging dynamic equilibrium of robustness on a neutral network. In this equilibrium, the average mutational robustness  $\bar{r}$ , the mean number of neutral neighbors the average sequence has in a population, attains a value that can be calculated analytically, using a graph-theoretical approach. In this approach, the neutral network of a secondary structure is represented by a graph. A graph is a mathematical object consisting of nodes (vertices) and edges. The nodes of this graph are the sequences in the network. Nearest (one-mutant) network neighbors are connected by edges. A convenient representation of such a graph is that of an adjacency matrix. In this matrix, which contains only zeroes and ones, rows and columns correspond to the nodes of the graph. An entry  $a_{ij}$  has a value of one if nodes (sequences)  $i$  and  $j$  are one-mutant neighbors on the neutral network, and zero otherwise. The average mutational robustness  $\bar{r}$  of a population that has reached equilibrium on the network is a simple characteristic of this adjacency matrix: It is equivalent to the largest eigenvalue of this matrix (562). This equilibrium mutational robustness  $\bar{r}$  is greater than the average number  $r$  of neutral neighbors of a randomly chosen sequence in the neutral network. Put differently, during evolution under selection maintaining a secondary structure, mutational robustness increases by an amount that is only a property of the structure itself—and of its associated neutral network—and not of the evolving population.



**Figure 4.3** Neutral network exploration. Shown is a two-dimensional projection of diversity in sequence space in a population of 2000 molecules after 135 rounds of replication with a mutation rate of 0.002 per nucleotide site and replication (248). Of the 2000 molecules, 631 differ in their RNA sequence, and 301 of these different sequences fold into a structure different from the target structure, a tRNA cloverleaf. Dots correspond to points in sequence space occupied by at least one member of the population. Large dots correspond to points occupied by more than four members of the population. Two dots are connected by a line if they are nearest neighbors in sequence space.

The two-dimensional projection of a 76-dimensional sequence space was obtained by computing a distance matrix  $M$ , whose entries calculate as  $m_{ij} = (d_{0i}^2 + d_{0j}^2 - d_{ij}^2)/2$ , where  $d_{0i}$  is the distance between sequence  $i$  in a population and the center of mass of the population, and  $d_{ij}$  is the Hamming distance between two sequences  $i$  and  $j$ . Through diagonalization of  $M$ , one can express the sequences in principal axes coordinates. The shown projection corresponds to the two main principal axes, which are associated with the two largest eigenvalues of  $M$ , and represent most of the variation in sequence space (248). After Figure 2 in (248). Copyright 1996 National Academy of Sciences, U.S.A.

The reason is that the structure completely defines the neutral network and thus the largest eigenvalue of the network graph. While it is impossible to make general statements about the extent to which robustness can increase during evolution, it is noteworthy that even very short structures can increase their robustness in this process. For example, the structure (((((. . . ))). .))) consists of only two short stacks. An average member sequence of the neutral network associated with this structure has 12 neutral neighbors. However, the average member of a large equilibrium population in the network has almost 15.7 neutral neighbors, reflecting an increase in robustness by 30% (562). Longer structures can experience severalfold increases in robustness (20).

All of this holds only for large populations or for small populations with high mutation rates. Specifically, it holds as long as the product of population size and mutation rate is much greater than one ( $N\mu \gg 1$ ). I discuss the reasons in chapter 16. If this condition is not met, e.g., if  $N\mu \ll 1$ , then mutational robustness does not increase in evolution. In other words, the average population robustness will not exceed that of a randomly chosen sequence in a neutral network, i.e.,  $\bar{r} = r$  (562).

### Have Biologically Important RNA Structures Evolved Increased Robustness Against Mutations?

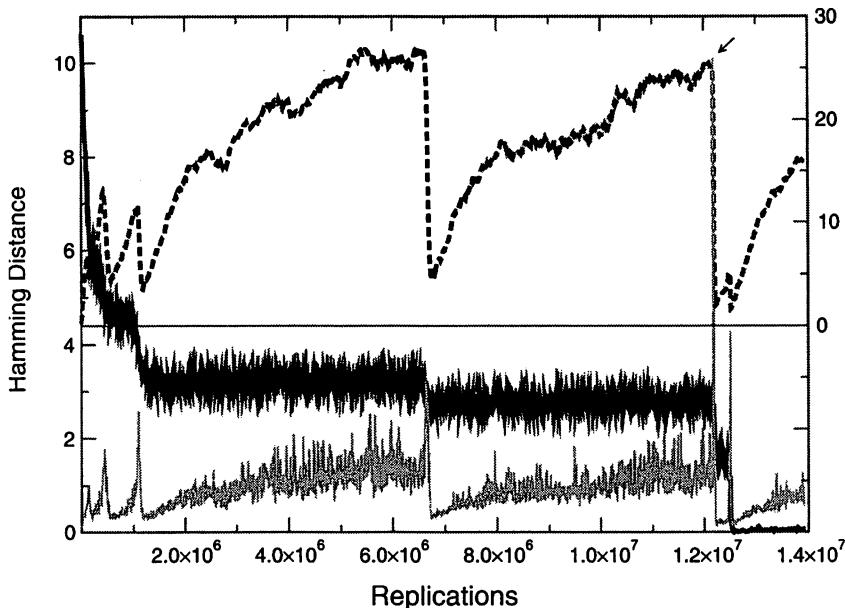
This is a largely unexplored question, except for one study, whose outcome is a tentative yes. In this study, Peter Stadler and I analyzed evolutionarily conserved secondary structures of several RNA viruses (590). Our analysis is based on the idea that conserved secondary structures are functionally important. Put differently, they are subject to selection to maintain their structure and can thus evolve increased mutational robustness under the above scenario. On one hand, viral secondary structures would seem ideally suited for such an analysis. The reason is that RNA viruses have very high mutation rates of up to  $10^{-3}$  per nucleotide position and replication round. They are thus likely to fulfill the above criterion necessary for the evolutionary increase of robustness. However, from a different perspective, viral secondary structures are much less than ideal. First, many of them are part of RNA regions that encode proteins. These regions are subject to severe evolutionary constraints other than to maintain their secondary structure, because they are also responsible for producing proteins essential to the virus. Second, although stability and robustness of important secondary structure is important, many viral secondary structures also need to be flexible enough to unfold for processes such as replication and translation. (The more flexible a sequence is in this sense, the lower

its mutational robustness, as I discuss below.) In consequence, natural selection may not favor maximally robust viral secondary structures. However, the requirement for a stable secondary structure may partially overcomes these constraints, as indicated by the following evidence for evolved mutational robustness among important viral RNA structures.

Our approach used multiple strains of three groups of single-stranded RNA viruses—dengue virus, hepatitis C virus, and HIV1—all of whose genomic RNA sequence has been completely determined. We determined short motifs (<150 nucleotides) of conserved secondary structures within each group by using a procedure to align the secondary structures of multiple viral genomes (235, 237). Pairs of viral genomes differed at between 19 and 23% of their nucleotides. Because altering as little as 10% of an RNA sequence's nucleotides at random changes its structure completely, most structural similarities between these viral genomes reflect selection maintaining secondary structure, a key requirement to increase mutational robustness.

In total, we determined 56 conserved secondary structures in these three viruses, and compared their mutational robustness to that of more than 100 nonconserved secondary structures. These nonconserved structures do not even occur in all viral strains and may thus play no important role in the viral life cycle. We compared the mutational robustness of conserved and nonconserved secondary structure motifs by generating all one-mutant neighbors of each motif and by determining by how much each mutant's minimum free energy structure differed from the motif's structure. (We used various measures of structural similarity, such as the Hamming distance I discuss above.) We found that conserved structures were more robust to mutations than nonconserved structures. Specifically, the one-mutant neighbors of sequences with conserved structures folded into structures that were between 10 and 50% more similar to the original motif's structure than the neighbors of sequences with nonconserved structure. In addition, conserved secondary structures were also more robust to mutations elsewhere in the viral genome, that is, outside the motif itself. Systematic differences between conserved and nonconserved structures in the number of paired bases, or the number of G-C base pairs, which confer greater stability, were not responsible for these differences in robustness.

The small differences in robustness we observed may be due to the other constraints these sequences are subject to. Studies on other RNAs may reveal greater evolutionary increases in mutational robustness. However, the ideal RNA molecule for such a study, subject to no constraints other than to maintain a maximally stable secondary structure, will be hard to find.



**Figure 4.4** Genetic diversity of RNA sequences during episodic evolution. Shown is the time course of evolutionary optimization toward the cloverleaf structure of a transfer RNA ( $n=76$  nucleotides), in a population of  $N=3000$  individual sequences with a mutation rate of 0.001 per nucleotide position and replication. Time is indicated on the horizontal axis in units of individual replications, where  $N=3000$  replications correspond to approximately one population generation. The middle plot (left vertical axis, black) indicates the mean Hamming distance  $d_H$  of the secondary structure of sequences in the population to the target cloverleaf structure. Note that step-like decreases in this distance are followed by long periods of no change. The upper and lower plots indicate two different measures of genetic variation among sequences in the evolving population. Specifically, the upper plot (dashed lines, right vertical axis) shows the average Hamming distance  $d_P(t, \Delta t)$  between pairs of sequences, where one member of the pair is taken from the population at time  $t$ , and the other member is taken from the same population at time  $\Delta t$  replication rounds later. The average is taken over all sequence pairs in the two populations. For small values of  $\Delta t$  (including  $\Delta t = 0$ ),  $d_P(t, \Delta t)$  is an indicator of the extension or the diameter of a population in sequence space. This also holds for the small value of  $\Delta t = 8000$  used to generate the data in the plot, especially considering the low mutation rate of 0.001, which will lead to only  $8 \times 76 = 590$  mutations among  $5.9 \times 10^5$  replicated nucleotides. The lower plot (solid gray lines, left vertical axis) shows the average distance  $d_C(t, \Delta t)$  between the mean nucleotide sequences (the “centers”) of two populations at time  $t$  and  $t + \Delta t$ . It is a measure of the distance that a population migrates in  $\Delta t$  replications (490). Specifically,  $d_C(t, \Delta t)$  is calculated from the population frequency  $\alpha_i^{(k)}$  of nucleotide  $i$  ( $i = A, U, G, C$ ) at

## The Halting Arrival of the Fittest

The previous section asked how evolution on a neutral network occurs if selection favors the secondary structure of this network. But what if one starts with a secondary structure that is far from this optimal structure? How does an evolving population find the optimal structure? This question has been approached systematically for small structures, such as the cloverleaf structure of transfer RNAs with  $n = 76$  nucleotides (161, 163, 490). The fact that RNA structures are robust to mutations holds the key to its answer.

Envision a large population of RNA molecules, each of which has the same sequence, randomly chosen in sequence space. All members of this population fold into the same structure, which may be very different from an optimal structure that natural selection might favor. Mutations cause the members of this population to diverge. Those mutants that fold into a structure closer to the target structure replicate faster, and thus come to dominate the population. The overall progress towards the target structure can be measured by the distance  $d_i$  of population member  $i$  to the target structure, averaged over all members of the population, i.e.,  $d = (1/N)(\sum_i d_i)$ , if  $N$  is the size of the population. The progress of the population toward the target structure can be divided into two phases. The first phase is characterized by rapid progress toward this structure, that is, rapidly decreasing population distance  $d$  to the target structure. The reason is that a small neighborhood around any one random sequence contains a great variety of structures that resemble the target more closely. In the second phase, long periods of evolutionary stasis alternate with short bursts of structural innovation, where the population rapidly approaches the target structure (Figure 4.4). In the long periods of stasis, the population does not approach the target. However, during these periods, the evolutionary search of the population does not simply grind to a halt. Instead, the population spreads—in exactly the manner I described above—through the neutral network associated with its current target structure. Occasionally, a member of this exploring population experiences a mutation that will bring its structure closer to that of

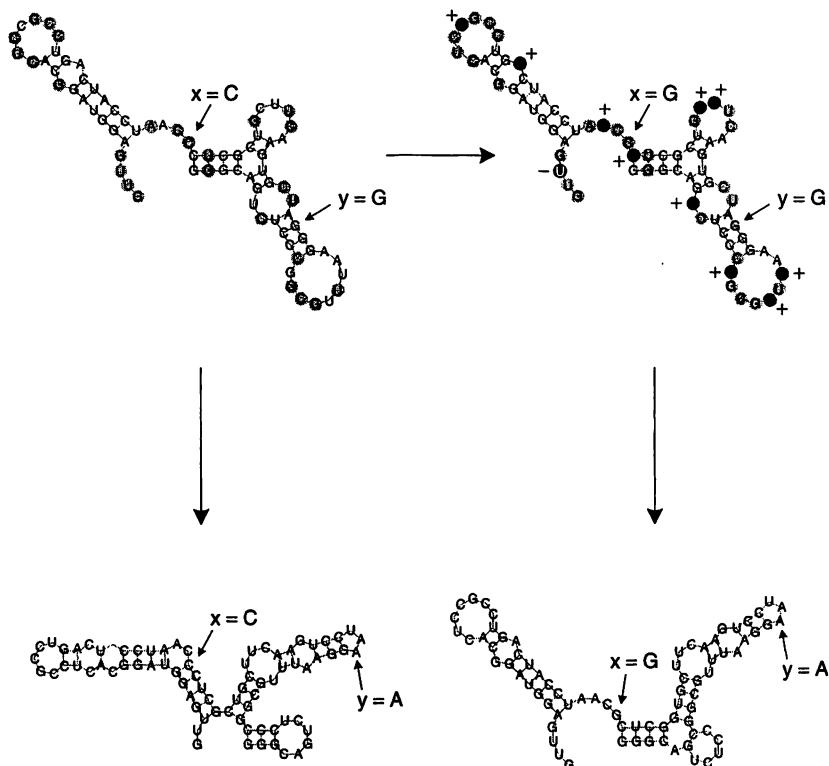
position  $k$  of the sequences in a population, where  $0 \leq \alpha_i^{(k)} \leq 1$ , and  $\sum_i \alpha_i^{(k)} = 1$ , and from the square-normalized distribution of nucleotides at position  $k$ , i.e., from  $\pi^{(k)} = \{\pi_A^{(k)}, \pi_U^{(k)}, \pi_G^{(k)}, \pi_C^{(k)}\}$ , where  $\pi_i^{(k)} = \alpha_i^{(k)} / \sqrt{\sum_{i=A,U,G,C} (\alpha_i^{(k)})^2}$ . With this notation,

$d_C(t, \Delta t)$  is defined as  $\Sigma_{k=1}^n = \sqrt{1 - \sum_{i=A,U,G,C} \pi_i^{(k)}(t) \pi_i^{(k)}(t + \Delta t)}$ . After Figure 10 in (490).

the target. This member and its descendants come to dominate the population, thus reducing the average distance  $d$  to the target structure. Put differently, in exploring the neutral network of the old structure, the population discovers the neutral network of a new structure closer to the target. It can then spread in this network until it discovers a structure even closer to the target, and so on.

This process of spreading through a neutral network and subsequent discovery of a gateway to a new network affects the genetic variation of a population and its location in sequence space, as shown in Figure 4.4 (490). The middle (black) graph in this figure shows a population's average distance to a target structure as the population approaches this structure. The figure shows four sharp reductions in this distance, which occur when the population "finds" four new structures that are increasingly closer to the target. The upper graph (gray, dashed) shows the average (Hamming) distance among sequences in the population, a measure of a population's diameter or extension in sequence space. Whenever a new structure is discovered, the population's diameter decreases dramatically, thus reducing genetic variation in the population. The reason is that the descendants of the sequence folding into the new structure, which are initially similar to each other, replicate faster than the older, genetically diverse, members of the population, which thus become extinct. A second consequence of the discovery of new structures is shown in the lower graph (gray, solid) of Figure 4.4. This graph shows an indicator of how fast the center of a population, a cloud of points in sequence space, changes position in the space. During exploration of a neutral network, this center changes position only slowly, a change that reflects the center's random drift as population members spread through sequence space (248). However, whenever a new structure is discovered, the population center shifts position very rapidly. The reason is that in that case, all but one of the subpopulations shown in the projection of Figure 4.3 become extinct. Only the descendants of the subpopulation that have stumbled upon a new structure continue the lineage.

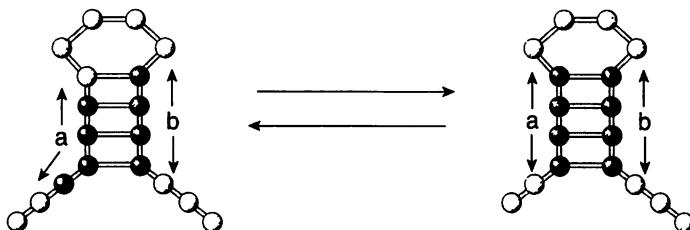
In sum, the evolutionary discovery of a specific RNA secondary structure proceeds in two alternating steps. First, a population explores the part of sequence space that folds into the same structure and accumulates great amounts of genetic variation in this neutral space. Second, one of the sequences in this population finally stumbles upon a better structure, at which point variation in the population is drastically reduced and the cycle repeats. Exploration of these neutral sequence variants is thus critical for subsequent innovation to occur. Individual sequences also contain a hint of this principle, as shown in Figure 4.5. The figure shows that a neutral sequence change can have multiple consequences. It affects the possibility of neutral and nonneutral changes at other nucleotide positions



**Figure 4.5** Neutral changes that make a difference. Bullets on the upper two secondary structures indicate neutral positions, that is, positions where at least one possible nucleotide change does not alter the structure. In the top left sequence, position  $x$  is neutral, because the  $C \rightarrow G$  substitution preserves the structure, as shown on the right hand side. However, neutral positions themselves change as a result of this neutral substitution. The dark bullets on the upper-right structure indicate positions that have become neutral (“+”) or stopped being neutral (“-”). The lower part illustrates that the neutral  $C \rightarrow G$  mutation at position  $x$  changes the consequences of altering  $A$  for  $G$  at the (nonneutral) position  $y$ , that is, the structural changes this nonneutral mutation causes. From Figure 3 in (161).

in the sequence, and the kind of structural changes that non-neutral changes can trigger.

Why are transitions between structures and neutral networks frequent in the initial stages of evolution towards a target structure, but then become rare later on? The answer lies in how mutations change RNA structure. Most of the initial structural changes, such as the lengthening or shortening of a stack by one base pair, are easily accomplished and frequent among the one-mutant neighbors of any sequence. In contrast, the

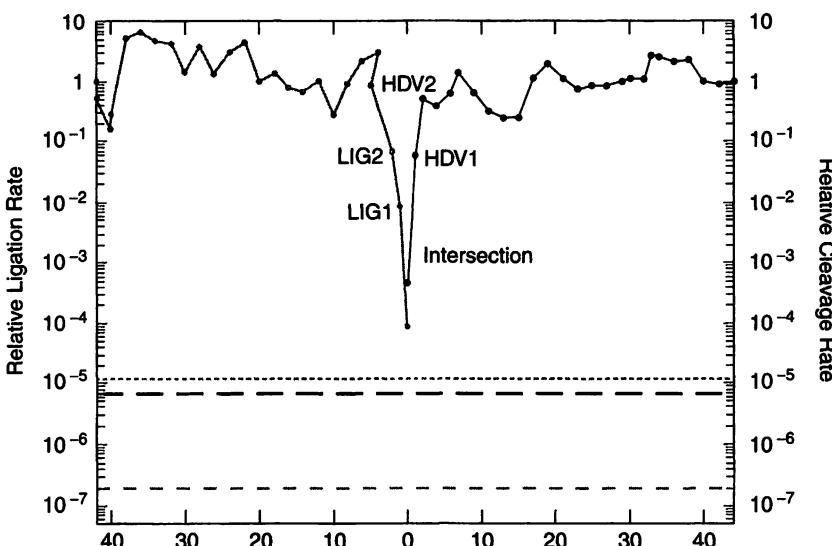


**Figure 4.6** An unlikely change in the secondary structure of an RNA molecule. In this change, the paired bases in a four-base-pair stack shift position relative to one another. This change leaves the size of the stack unchanged but can change the adjacent loop. Such unlikely structural changes, some of which can change structures more drastically, characterize the sharp transitions toward a target structure during the episodic evolution shown in Figure 4.4. After Figure 6A in (161).

structural changes that occur later on are much less easily realized. They include large, discontinuous transformations, such as the formation of a new stack from an unpaired region, or shifts in the paired bases that are part of a stack (Figure 4.6). Changes such as a loop–stack transition require previously unpaired and noncomplementary base sequences to become paired and complementary and thus can occur only at certain, rare points on a neutral network. Such changes are key to finding a complete match to a target structure (161, 163).

## A Ribozyme Experiment

Both minor evolutionary variation and radical innovations in RNA structure amount to transitions between adjacent neutral networks (161, 163, 164). This general observation required the analysis of millions of secondary structures, which would make its experimental corroboration seem highly unlikely. However, a very illuminating direct proof of this principle exists. It shows that ribozymes—catalytic RNAs—with vastly different catalytic activities, structures, and sequences can inhabit adjacent neutral networks (487). The subject of this study are one natural and one synthetic ribozyme. The natural ribozyme is encoded by the hepatitis delta virus, a human pathogen with a single-stranded RNA genome. This ribozyme is responsible for a self-cleavage reaction that is necessary to complete the viral life cycle. The synthetic RNA is the class III self-ligating ribozyme, which joins an oligonucleotide substrate to its own 5' end and was isolated in the laboratory from a pool of random RNAs. The sequences of these two ribozymes have no more than the 25% sequence



**Figure 4.7** A series of neutral mutations connects a hybrid ribozyme with the two ribozymes it is derived from. The horizontal axis shows the distance (in single nucleotide changes) of the hybrid ribozyme (positioned at distance 0) from its ancestors, a ligase (toward the left) and a self-cleaving ribozyme (toward the right). The vertical axis shows the reaction rate of each ribozyme (left = ligation, right = self-cleavage) as a fraction of the respective ancestor. The relative rate for the uncatalyzed ligation reaction is indicated by the short-dashed line (ligation with formation of a 2'-5' linkage) and the dotted line (ligation with formation of a 3'-5' linkage). The relative rates of the uncatalyzed cleavage reaction are indicated by the long-dashed line. From Figure 3A in (487).

identity expected by chance alone, and no structural similarities that might favor the nearness of their respective neutral networks (487). Nevertheless, Schultes and Bartel (487) were able to design an RNA molecule that simultaneously has both catalytic activities, that of the self-cleaving ribozyme and that of the ligase. This sequence is more than 40 mutational steps away from both the prototype ligase and from the prototype self-cleaving ribozyme. Its activity is substantially lower than that of the prototype ribozymes (Figure 4.7), but still 70 times higher than that of uncatalyzed RNA cleavage and 460 times higher than that of the uncatalyzed ligase reaction. Importantly, this hybrid sequence can be linked via a series of point mutations to both prototype ribozymes, without reducing its activity. Two point mutations into the direction of the ligase restore near wild-type levels of ligase activity, and two point mutations in the other direction restore near wild-type levels of the self-cleavage activity. The remaining, approximately 40 point mutations in either direction

are neutral in the sense that they keep the catalytic activity close to the level of the prototype ligase and self-cleaving ribozyme (Figure 4.7). By constructing a hybrid ribozyme and constructing a path through sequence space back to its ancestors, this work shows two things. First, RNAs with very different functions can be near each other in sequence space. Second, each of these RNAs is part of a vast space of alternative solutions to the same catalytic and structural problem.

### Mutational Robustness and the Structure Repertoire of a Sequence

All the material I have discussed thus far regards mutational changes in a sequence's minimum free energy structure. However, the minimum free energy structure is only the most stable among many structures that a sequence can assume at ambient temperatures. The number and identity of its alternative structures is correlated with the effects of mutations on the minimum free energy structure, a correlation that will become important in chapter 17. The first aspect of this correlation regards how mutations in a sequence change the sequence's minimum-free energy structure: As shown by Ancel and Fontana, many of the minimum free energy structures in one-mutant neighbors of a sequence are identical to suboptimal structures of the sequence. In other words, the repertoire of structures a sequence can adopt in response to thermal noise resembles the minimum free energy structures of its mutants (20, 161).

The second aspect of this correlation is that mutationally robust sequences tend to show a reduced repertoire of suboptimal structures. A representative example is a structure of 78 nucleotides with 40 paired bases studied by Ancel and Fontana (Figure 4.15 in ref. (20)). A randomly chosen sequence from the neutral network folding into this minimum free energy structure contains 574 suboptimal structures within a  $5kT$  interval of the minimum energy structure at  $37^\circ\text{C}$ . The sequence itself spends only 3% of its time in the minimum free energy structure, and many of its suboptimal structures are drastically different from the minimum free energy structure. This large repertoire of suboptimal structures is accompanied by comparatively little mutational robustness of the sequence: Mutations at only 27 of the 78 nucleotide positions leave the minimum free energy structure unchanged, and only 18.4% of the total number of  $3 \times 78 = 234$  possible single nucleotide changes are neutral. Contrast this with the mutational robustness of a sequence from a population that has evolved structural robustness by finding regions of high robustness in this structure's neutral network, as explained above. This sequence has only 27 alternative structures and spends 34% of its time in

the minimum free energy structure. Concomitantly, the number of positions allowing neutral mutations has increased to 43 of 78, and now a much greater fraction (41%) of all mutations are neutral (20).

Thus far, I have only discussed studies of RNA robustness where selection favors an RNA's minimum free energy structure. Such studies usually ignore the number and stability of other suboptimal structures. One can, however, also apply selection to render the minimum free energy as stable as possible, thus disfavoring sequences with many suboptimal structures. The correlation between robustness and the number of suboptimal structures suggests that doing so will reduce mutational robustness even further. This is indeed true (20). In terms of the above example, if selection to increase the time spent in the minimum free energy structure is applied to the original sequence, the number of suboptimal structures decreases to four (from originally 574). A sequence thus evolved spends 67% of its time in the minimum free energy structure. In addition, its few suboptimal structures are almost identical to the minimum free energy structure. Concomitantly, 68 out of 78 sequence positions come to allow neutral mutations, and the total percentage of mutations that do not change the minimum free energy structure increases to 45%.

Thus, natural selection can favor an RNA structure in several similar ways that increase mutational robustness. Selection can favor sequences that have a particular minimum free energy structure, it can favor sequences that spend much time in this structure, or it can favor sequences with a small repertoire of suboptimal structure. In all cases, mutational robustness will increase. In biological systems such as RNA viruses where selection may have increased mutational robustness (590), it may thus be difficult to delineate exactly how it did so. The extreme robustness and absence of structural plasticity that can evolve through natural selection also point to a downside of neutrality: Even though necessary for structural innovation, robustness in its extreme can hinder innovation. Sequences that have been subject to a reduction in their repertoire of suboptimal structures become concentrated in a part of a neutral network where most mutations either have no effect or change the minimum free energy structure to a very similar structure. Under these circumstances, structural innovation is no longer possible (20, 161).

In sum, frequent RNA structures are intrinsically robust to mutations and inhabit vast connected parts of sequence space within which many mutations are neutral. Their robustness can further increase through natural selection, if selection favors the structure's persistence. Such robustness, and the ensuing large numbers of neutral mutations, is also essential for structural innovations. However, robustness can become confining if selection also curtails a sequence's repertoire of suboptimal structures.

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## Proteins and Point Mutations

What is robust? The three-dimensional structure and function of proteins.

What is it robust to? Changes in a protein's amino acid sequence.

The main factor hindering the experimental analysis of robustness in RNA structure is the difficulty of determining this structure experimentally. Powerful computational techniques for RNA structure prediction have alleviated this problem. The situation in proteins is the converse. There is abundant experimental data on protein structures and their robustness. In contrast, fewer systematic computational analyses of problems that are difficult to address experimentally exist for proteins. However, where they ask similar questions, experimental and computational analyses of protein structure have often yielded answers similar to those obtained for RNAs. Specifically, such analyses show that the structure and function of many proteins are very robust to changes in individual amino acids; they demonstrate that there are many alternative ways, a vast neutral space, of building proteins with similar structure or function; and they show that robustness can increase through incremental evolution in this space. Here I examine the evidence for these statements, as well as the many remaining open questions in this area.

Many analyses of protein robustness have focused on a protein's tertiary structure, the arrangement and folding of a protein's amino acid sequence in three-dimensional space. While the analysis of tertiary structure is an improvement over the analysis of secondary structures that dominates RNA work, tertiary structure is not always the best possible proxy for protein function. This holds especially for enzymes. An enzyme's catalytic site is formed by few surface amino acids that are responsible for the enzyme's substrate specificity and reaction chemistry. Not surprisingly, then, enzymes with very similar tertiary structures can adopt a great variety of catalytic functions (468, 552, 554, 555). Thus, at least for some proteins, the tertiary structure just provides a backbone, on top of which different functions can be built by subtle modifications. Despite this

caveat, disrupting a tertiary structure will also disrupt the function of the protein. Thus, a properly formed tertiary structure is at least a necessary ingredient for proper protein function. This is why the robustness of a protein's tertiary structure, similar to that of RNA secondary structure, deserves study in its own right.

### Experimental and Comparative Studies of Protein Robustness

Experimentally, the robustness of proteins can be explored in two principal ways that are already familiar from chapter 1. First, one can experimentally perturb a protein by changing one or more of its amino acids and assay whether its structure or function changes. Such mutagenesis studies suggest that the function of many proteins is insensitive to the vast majority of possible single amino acid changes. A case in point is a study of the lysozyme of bacteriophage T4, which is necessary for host cell lysis and completion of the viral life. Rennell and coworkers substituted 13 different amino acids at each of 163 amino acid positions of the protein, for a total of 2015 amino acid changes (461). Only 16% (328) of these variants affected T4 function severely, as indicated by the reduced ability of phages to form plaques of lysed cells on continuous lawns of bacteria. Of 163 amino acid positions 89 were able to tolerate any of the 13 amino acids without disrupting lysozyme function. The minority of amino acid positions that were sensitive to changes fell in two categories. The first category comprises amino acids in the hydrophobic core of the protein. This core is more rigid than the surface and moves little in response to thermal noise (361, 461). The second category includes few surface amino acids that are thought to be directly involved in catalysis (461).

In a similar study on a  $\beta$ -lactamase of *E. coli*, Huang and collaborators found that 84% of this enzyme's 263 amino acids can suffer point mutations without severely impairing the enzyme's ability to confer antibiotic resistance (245). This result is based on a mutagenesis method that not only generates single nucleotide and thus single amino acid substitutions, but instead, randomizes all nucleotides in two, three, or four consecutive codons. It can thus generate not only all one-mutant neighbors of a protein at one amino acid position, but up to four-mutant neighbors at consecutive amino acid positions. Many of the functional  $\beta$ -lactamases emerging from this procedure were two-, three-, and four-mutant neighbors of the wild-type  $\beta$ -lactamase, thus showing that even distant neighbors of a protein can still be functional.

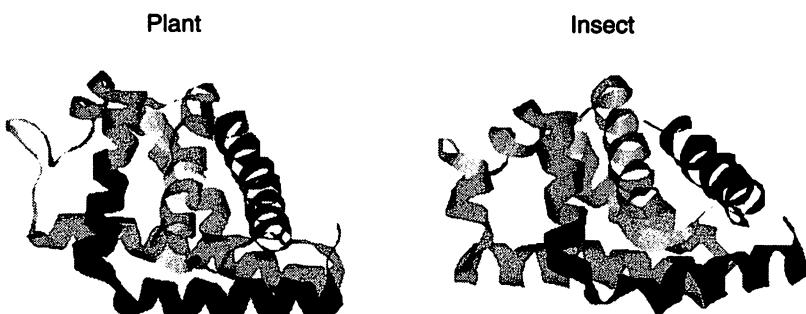
Many other studies tell a similar story. Of 1634 single amino acid substitutions in the *E. coli lac* repressor, a transcriptional regulator, 55% do not lead to loss of the protein's function (289); most of 300 mutant human

hemoglobins are fully functional (604); and most positions in a protease of the AIDS-causing virus HIV-1 (339) as well as in the amino terminal domain of phage  $\lambda$  repressor—a prokaryotic transcriptional regulator—can tolerate many amino acid changes (58).

A second type of analysis complements this picture of extensive robustness in protein function. This type of analysis compares proteins that have a common ancestor and that have similar function in multiple species. It asks how similar their structures and amino acid sequences are. While mutagenesis experiments explore the immediate neighborhood of a functional protein in sequence space, this second approach examines the end points of an evolutionary exploration of this space, an exploration that preserved a protein's function, sometimes over more than a billion years. If this exploration usually stayed within a tiny region of sequence space, one would conclude that only little change in amino acid sequence is permissible if a protein's function is to be preserved. If so, robustness would be very limited for most proteins. However, this is generally not the case. Many proteins with similar functions and structure have very different amino acid sequences.

One of the most comprehensive comparative analyses regards the globin fold, a structure characteristic of oxygen-binding proteins. The most prominent examples of proteins with this fold are vertebrate myoglobin and hemoglobin. These proteins have numerous distant relatives in many vertebrates, invertebrates (mollusks, arthropods, and annelids), and even plants, where they bind oxygen to facilitate nitrogen fixation. Not all of these proteins have absolutely identical biochemical function—they bind oxygen with different affinities and kinetics—but they all bind oxygen (183, pp. 38–40).

The tertiary structures of even distant globin representatives are very similar (Figure 5.1). For instance, the three-dimensional structure of whale myoglobin and the hemoglobin of the clam *Lucina pectinata* can be superimposed almost exactly (463). Despite great structural similarities, however, the amino acid sequences of these globins are very different. The clam hemoglobin has only 18% amino acid identity to vertebrate hemoglobin. In a study of 6 hemoglobins from plants and animals, Aronson and Hendrickson (25) found that as few as 12.4% of amino acid residues were identical between any protein pair. In addition, despite their similar tertiary structures, only 4 out of 97 amino acids in the protein were unchanged in all of the proteins. Amino acid similarities this low raise the question of whether different globins have arisen multiple times independently in evolution and do not share a common ancestor. Phylogenetic information argues against this possibility, at least when animal and plant globins are considered separately. For instance, amino acid similarities of globins among different animal species reflect the evolutionary relatedness

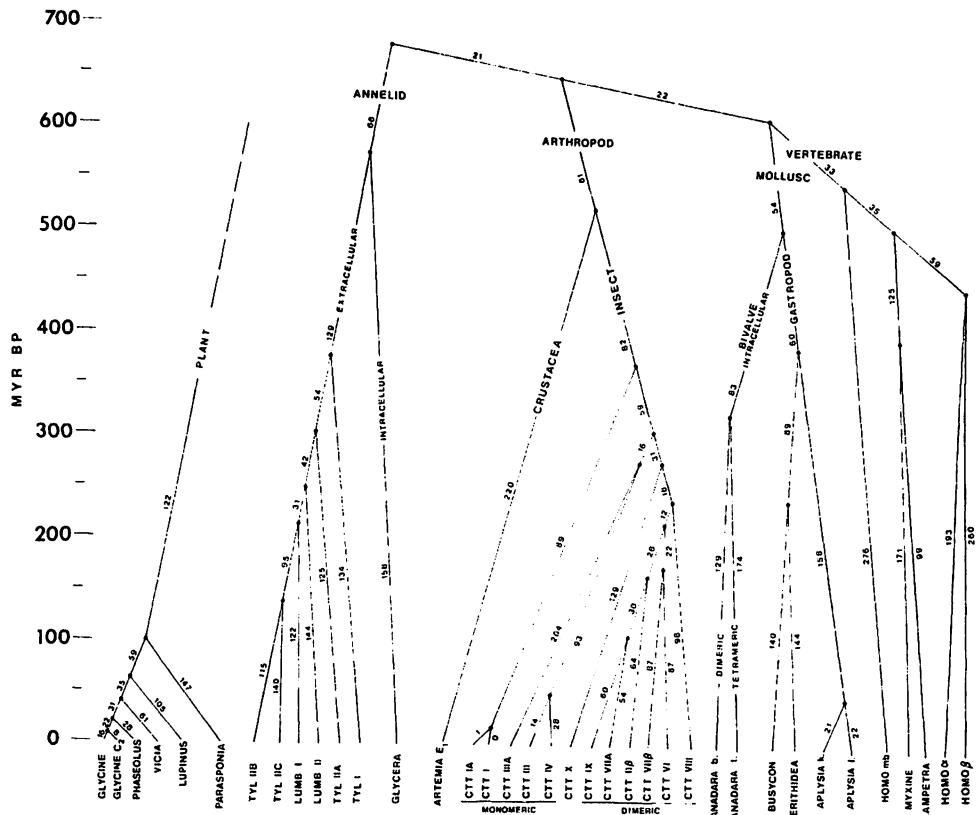


**Figure 5.1** Two globin molecules with very similar structures but little amino acid sequence similarity. The left image shows a ribbon diagram of root nodule hemoglobin of the lupine *Lupinus luteus* (26). The right image shows the structure of an insect hemoglobin, that of the midge *Chironomus thummi thummi* (521). Both structures consist of 7  $\alpha$ -helices in a spatially very similar arrangement. The root mean square difference in the position of 97 alpha carbon atoms in the backbone of these helices is 2.88 Å (25). Only 15.5% of the amino acids are identical in the seven conserved helices (25). The globins shown correspond to entries 2LH3 and 1ECO of the protein data base PDB (43).

of the species, such that more distantly related species harbor globins with more dissimilar amino acid sequences (198) (Figure 5.2).

### Protein Domains Tell the Same Story as Whole Proteins

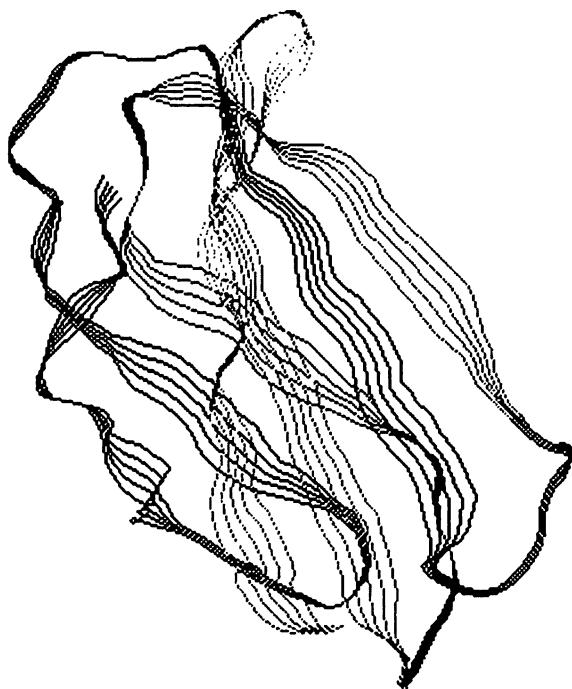
With increasing availability of protein structures, it has been realized that many proteins are assemblies of smaller parts or structural domains. A domain is a distinct, compact, and stable unit of protein structure that folds independently of other such units. It often also has a unique function. Because many proteins consist of multiple domains with distinct structures, structural comparisons are focusing increasingly on domains themselves. Such comparisons reveal patterns very similar to those of whole proteins. A case in point is the fibronectin type III domain (Fn3) (Figure 5.3), which adopts a tertiary structure similar to one also found in immunoglobulins. It is widespread in animals and has also been found in some bacteria (52). The proteins in which it occurs include extracellular matrix proteins such as fibronectin—involved in processes as diverse as tissue repair, blood clotting, and cell migration—intracellular proteins, and many kinds of membrane receptor proteins, such as the human growth hormone receptor. Despite their highly similar tertiary structures, amino acid sequence similarities of Fn3 domains in different species are as low as 9% (52).



**Figure 5.2** Evolutionary relationships among globins from 6 plants, 26 invertebrates, and 5 vertebrates. The phylogenetic tree shown is based on amino acid alignments and a maximum parsimony algorithm. The numbers along each tree branch represent the numbers of substitutions that took place along the branch. The left vertical axis represents time in million years before present (MYR BP). CTT refers to the midge *Chironomus thumi thumi* and its various globins. TYL stands for *Tylorrhynchus*, a polychaete worm, and LUM for *Lumbricus* (earthworm). From ref. (198).

### More Than Anecdotal Evidence

Examples such as those of globins and the Fn3 domain show that evolutionary explorations of sequence space in the billion-year-long history of eukaryotes can range very far without compromising a protein's structure. However, piling example upon anecdotal example to illustrate that vastly different proteins can adopt similar tertiary structures does not show how representative this phenomenon is of all proteins. The reason



**Figure 5.3** Ribbon diagram of the fibronectin type III domain. The domain consists of a three-stranded  $\beta$ -sheet and a four-stranded  $\beta$ -sheet. Data from (324). Image from (43).

is that counterexamples exist as well. These are proteins with conserved structure and very similar sequences in widely divergent organisms. A case in point are structural proteins like actins, tubulins, and histones, which may show up to 98% sequence identity in organisms as dissimilar as humans and plants (119). Perhaps these proteins are the rule rather than the exception?

Only statistical surveys of many protein structures can answer this question. Such surveys have become possible with the availability of large protein structure databases, and with techniques to identify proteins with similar tertiary structures. However, such surveys are still difficult, because many proteins with similar structures are located close together in sequence space. The reason is simply that they derive from a recent common ancestor—a single point in sequence space—from which they have diversified. This means that proteins with the same structure are not unbiased representatives of all sequences that fold into the structure.

In a statistical survey that alleviates this problem, Rost (467) first identified 272 proteins with dissimilar tertiary structure in the protein

data bank, a database of thousands of protein structures at atomic resolution (43). Using each such protein with a unique structure as a reference protein, he identified all other proteins in the database that were so dissimilar to the reference protein in amino acid sequence (<25% identity) that their common ancestry with the reference protein is doubtful (120). Many of these proteins are, however, very similar in structure to the reference protein. Because of their low sequence similarity to the reference protein, such proteins are a less biased sample of the part of sequence space that folds into the same structure. Strikingly, such proteins with similar structure shared on average only 8.5% of their amino acids, much fewer than the 25% threshold preimposed on the analysis. This number is only slightly higher than the 5.6% amino acid identity between any two proteins chosen at random from the database. Other surveys of different protein structures yield a similar picture: many protein structures can be realized by very different amino acid sequences (552, 554).

In sum, as a rule with some exceptions, proteins with the same structure occur in very distant parts of an enormous sequence space. Juxtapose this finding with the experimental evidence for the mutational robustness of proteins and the following picture emerges: Different solutions to one and the same structural problem can be realized in very different parts of sequence space. These parts can be connected through a series of single amino acid exchanges that leave protein structure intact. Proteins such as hemoglobins, whose evolution is well studied, have explored this space extensively.

## Background on Computational Approaches

The evidence I just summarized leaves a number of questions untouched. One of them is whether the extraordinary robustness of protein function and structure has involved in response to natural selection favoring such robustness. Fortunately, computational methods to study folding in simple model proteins yield insight into these questions and complement existing experimental work. Before going into the relation between such experimental and computational evidence, some background material will be useful.

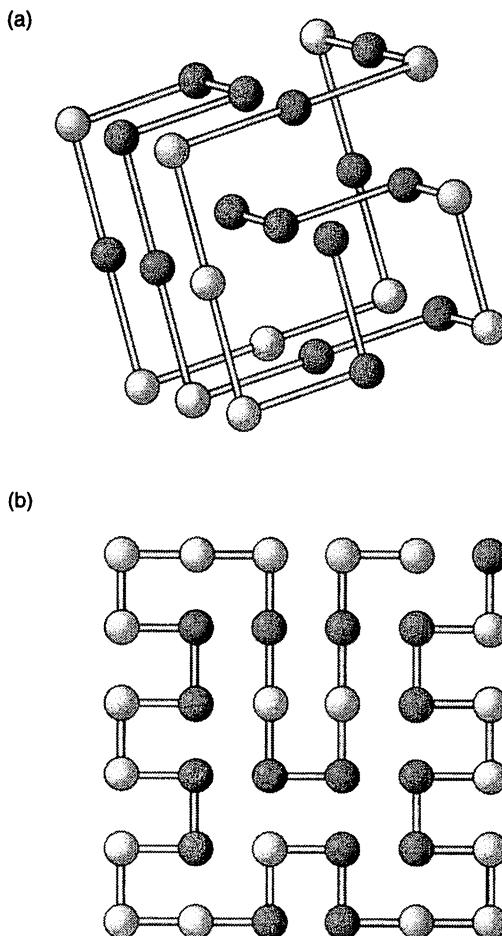
Computational models of protein folding rest on the notion that proteins will fold into a native conformation or tertiary structure that is compact in space and that minimizes the protein's free energy  $E$ . An important contribution to this free energy are noncovalent interactions—hydrogen bonds, hydrophobic interactions, and ionic bonds—between amino acids that are not adjacent in the amino acid chain (60). Some such interactions

are favorable and reduce the protein's free energy, whereas others are unfavorable and increase it. The native conformation has the largest number of favorable and strong interactions. It is usually also a compact conformation, in the sense that amino acids are densely packed in it. The principal obstacle to brute force calculation of this native conformation is the astronomical number of nonnative conformations any one protein can adopt. Computational approaches take various shortcuts to alleviate this problem.

An important class of spatially explicit yet tractable models of protein folding are lattice proteins (53, 55, 64, 66, 73–76, 97, 117, 118, 139, 204–206, 330, 335, 337, 475, 476, 508, 540, 566, 612). A paper from Lipman and Wilbur (337) spawned a now mushrooming industry of evolutionary studies using such protein models. In lattice proteins, folding is constrained in one important way: In the native conformation individual amino acids can assume positions only on a discrete grid—a lattice. This grid can be either two dimensional or three dimensional (Figure 5.4). It can also have a variety of geometries (triangular, square, etc.) distinguished by the number of neighbors each amino acid has on the lattice. The advantage of this discrete representation of tertiary structure is that all possible tertiary structures of an amino acid chain can be enumerated. There are, for example, fewer than  $10^5$  possible tertiary structures that completely fill the three-dimensional cubic lattice of Figure 5.4 (335). To identify the most thermodynamically stable among these structures, lattice protein models make several assumptions. For instance, they may represent proteins as chains of only two types of amino acids, polar (P) and hydrophobic (H). Any protein's amino acid sequence is then completely determined by choosing one of these types for every position along the chain. When folded compactly, that is, on the lattice, a protein's free energy  $E$  can then be calculated with the following expression:

$$E = \sum_{i,j, i < j} E_{a_i a_j} \delta(i, j) \quad (5.1)$$

Summation extends over all pairs of different sites  $(i, j)$  on the lattice.  $\delta(i, j)$  is equal to zero if two sites on the lattice are not nearest neighbors or if they are occupied by amino acids that are adjacent on the amino acid chain. This means that such sites do not contribute to the free energy.  $\delta(i, j) = 1$  only for neighboring sites on the lattice that are occupied by nonneighboring amino acids on the chain.  $E_{a_i a_j}$  is the contribution that interactions between such amino acids  $a_i$  and  $a_j$  make to the total free energy  $E$ . If there are only two types (H and P) of amino acids, this interaction energy can assume only three values,  $E_{HH}$ ,  $E_{HP}$ , and  $E_{PP}$ . Their values can be chosen to



**Figure 5.4** Lattice proteins. A protein is represented by a chain of black and white beads, corresponding to hydrophobic and hydrophilic amino acids. (a) A protein of 27 amino acids folded onto a  $3 \times 3 \times 3$  three-dimensional cubic lattice. (b) A protein of 36 amino acids folded onto a  $6 \times 6$  two-dimensional cubic lattice. From Figure 1 in (335).

mimic features of actual proteins, e.g., that amino acids of the same type interact preferentially (e.g.,  $E_{HP} > E_{PP}, E_{HH}$ ) (335). Exhaustive enumeration of all possible lattice tertiary structures and their free energies permits calculation of the most stable structure, the structure with the smallest minimum free energy  $E$ .

Even this simplest of models captures important aspects of protein folding. For example, the 20 naturally occurring amino acids can be subdivided

into two distinct groups (H and P) according to their affinity for water. It is the tendency of hydrophobic amino acids to avoid water that drives different proteins to fold into compact shapes with a core of hydrophobic amino acids (60). Some proteins can even be designed or redesigned only by choosing suitable hydrophobic and polar amino acids along the chain (88, 269).

Extensions of this model can capture increasingly subtle aspects of protein folding. Such extensions include larger alphabets of up to 20 amino acids, empirically determined interaction energies for these amino acids, and modifications to incorporate the effects of the solvent in which the protein folds (65). The disadvantage of some such extensions is their computational cost, a cost that is often controlled by making other simplifying assumptions that may be problematic. There is also a great variety of conceivable model extensions, which is responsible for conflicting results between different studies, a major weakness of protein folding models (65). Comparing the results of different approaches becomes especially problematic when comparing lattice models to other models of protein folding. One class of such models folds simple model proteins without confining them to a lattice (399, 406). Another class of models does not represent protein conformations in space, but only their free energies (28, 64, 66).

As stated above, most models of protein folding rest on the thermodynamic hypothesis of protein folding, which states that a protein's native conformation is its minimum free energy conformation (21). This hypothesis has been criticized on the grounds that a protein cannot possibly explore the vast space of all its possible conformations to find the minimum free energy conformation (648). An alternative is kinetic folding, where parts of a protein fold in a well-defined temporal order, an order defined by the protein's amino acid sequence, and by the order in which this amino acid sequence emerges from a ribosome. Put differently, if the temporal trajectory of a folding protein through the space of conformations—its folding kinetics—determines the protein's final structure, then the minimum free energy structure may not be accessible. This tension between the thermodynamic and kinetic folding hypotheses is alleviated by the suggestion that proteins do explore many conformations, but not randomly. Instead, their minimum free energy structure may be surrounded by a “folding funnel” of similar structures with higher free energies (330, 406, 648). This folding funnel guides the folding kinetics through states of increasingly lower free energy to the minimum free energy structure. Thus, while some proteins may adopt native structures different from their minimum free energy structure (41), thermodynamic and kinetic folding are not in contradiction for most proteins.

## Insights from Computational Studies

Before surveying what computational approaches can tell us about protein robustness, I will briefly revisit some key insights about RNA structure from chapter 4 and integrate them with related information on protein structure. At the root of robust RNA secondary structures is the huge discrepancy between the number of RNA sequences and the number of possible structures. Also, far from being uniform, the distribution of the numbers of sequences folding into any one structure is highly skewed for RNA. There are many rare structures, structures with few sequences folding into them, and few frequent structures, but the vast majority of sequences fold into these frequent structures. The frequent structures are especially robust against mutations.

Similar patterns exist for protein tertiary structures. First, the discrepancy between the space of possible amino acid sequences and the number of possible protein structures is even more flagrant than for RNA structures. Even for small proteins of 100 amino acids, there are  $20^{100}$  possible amino acid sequences. This contrasts with the number of possible protein folds, which varies according to the estimation method but is less than 10,000 according to all available estimates (89, 207, 416, 598, 621, 641, 642). Second, empirical estimates of the distribution of folds also suggest a skew similar to that observed for RNA (301). Specifically, there is a small number of “frequent” tertiary structures (301), such as the TIM-barrel, named after the enzyme triosephosphate isomerase where it was first discovered, or the Rossman fold, a tertiary structure found in nucleotide-binding proteins (60, 395). Such tertiary structures are frequent in the sense that they occur in multiple families of proteins. Members within a family have significant amino acid sequence similarity and thus a common ancestor. However, little such similarity exists among families, raising the possibility that such folds may have originated multiple times independently and in different regions of sequence space. Conversely, the majority of protein structures are “unifolds,” realized by only one family of proteins with the same, unique evolutionary origin (301). While this pattern of structure distribution indicates a skewness similar to that found for RNA structures, it does not strictly answer how rare and frequent structures are distributed in sequence space. Specifically, protein structures realized by the living need not be an unbiased sample of all possible structures in sequence space, but may represent a small subset of all structures with desirable features.

With this shortcoming of empirical data in mind, it is reassuring that work on simple model proteins also argues for the skewed distribution of protein structures (53, 335). For instance, Li and collaborators examined

all possible sequences of hydrophobic and polar amino acids in three-dimensional ( $3 \times 3 \times 3$ ) and two-dimensional lattice proteins (various lattice sizes) that have a unique minimum free energy structure. They found, first, that there are many fewer structures than sequences. For instance, for the three-dimensional case, the average structure is realized by 62 of the  $2^{(3 \times 3 \times 3)} = 1.3 \times 10^8$  possible sequences. Second, as in the case of RNA, the distribution of structures is highly skewed. Most structures are attained by many fewer than this average number of sequences, but a small number of frequent structures are attained by many more (up to 3794) sequences. These frequent structures also have symmetries that are reminiscent of  $\alpha$ -helices and  $\beta$ -strands in real proteins (335). In addition, the most frequent structures are thermodynamically more stable than other structures. That is, the difference in the minimum free energy (5.1) of the most stable structure and the structure with the second-lowest free energy is larger than for rare structures. Moreover, these most frequent structures can tolerate substitutions of amino acids at a large fraction of their positions. For example, for the most frequent three-dimensional structure, the probability of finding a hydrophobic or polar amino acid is close to one-half for 11 of their 27 positions. In other words, whether an amino acid is polar or hydrophobic does not affect the native structure at these positions. Many of these variable positions occur on the surface of the lattice, analogous to surface amino acids of real proteins. In addition, the most frequent lattice structures can be realized by highly dissimilar amino acid sequences (335).

These results have to be taken with a grain of salt, especially because of the small size of the amino acid "alphabet" (two amino acids), and because other aspects of protein folding are sensitive to the number of different amino acids and their interaction energies (65). However, they do show a striking similarity to patterns seen in real proteins. In addition, they suggest that frequent protein structures are both robust against mutations and thermodynamically stable.

Can protein structures evolve mutational robustness in the same sense as RNA? That is, can evolving populations of proteins under selection to maintain a structure accumulate in regions of high mutational robustness, where many one-mutant neighbors fold into the same structure? In a study on lattice proteins addressing this question, Bornberg-Bauer and Chan analyzed the evolution of chains of 18 monomers that fold onto a two-dimensional lattice (55). The model involves two types of monomers (H and P) and two different interaction energy models. The first of them rewards interactions between hydrophobic amino acids, but does not contain a contribution of other interactions to the free energy of a conformation. The second model favors interactions between like amino acids (H-H, P-P) and disfavors interactions between unlike amino acids (H-P). Both models yield qualitative similar results.

Most importantly, the sequences folding into the most frequent structures form sets whose members are connected through single amino acid exchanges that leave the structure unchanged. Not necessarily all sequences folding into the same structure are connected in this sense. Whether they are, that is, whether they form a single neutral network of sequences, depends on the frequency of the structure and on the model of interaction energies used (53, 55). In general, as for RNA, the more frequent a structure, the more likely it is that all its sequences are connected. Unlike for RNA, however, a quantitative criterion for connectedness of all sequences is currently not available for lattice proteins.

When selection to maintain a structure confines an evolving population of proteins to one connected set of neutral neighbors, the population will follow an evolutionary trajectory identical to that observed for RNA structures (562). That is, its members will accumulate in regions of sequence space where sequences have many neutral neighbors and thus high mutational robustness. For simple two-dimensional lattice proteins, these are regions centered around one or few maximally robust sequences, surrounded by increasingly distant and less robust neighbors with the same structure (53, 55). As observed for RNA, the population will reach a dynamic equilibrium of robustness at which the mean number of neutral neighbors of a protein is identical to the largest eigenvalue of the adjacency matrix of the graph describing the network (see chapters 4 and 16). In this equilibrium, the robustness of an average population member will be greater than the robustness of a sequence chosen at random from the neutral network. The attainable extent of robustness in this process is proportional to the number of sequences in a neutral network (635). Put differently, the more frequent a structure, the more robust it can become in evolution.

This identical pattern of protein and RNA structure evolution is no coincidence. In both cases, evolution is confined to a connected subset of equivalent sequences—those folding into the same structure. In both models, mutations can interconvert these sequences. The principles of structure formation, which are different for RNA and proteins, are secondary to the evolutionary process. In contrast, the topology of the network of neutral neighbors is of primary importance.

## Thermodynamic Stability of Proteins

A more refined picture emerges if one not only considers the minimum free energy structure itself, but also measures its thermodynamic stability relative to other, suboptimal structures. There are various correlated measures of thermodynamic stability, the simplest among them the energy

gap, the difference in free energy between a minimum free energy structure and the next-most stable structure (64, 65).

The thermodynamic stability of a sequence is correlated with its mutational robustness (53, 55). More specifically, the greater a sequence's mutational robustness, that is, the more neutral neighbors it has, the higher is its thermodynamic stability. Thus, when a population of sequences evolves increased mutational robustness of its member sequences, it also evolves increased thermodynamic stability, and vice versa. As a by-product, the probability that a mutation does not decrease protein stability can also increase during evolution. Taverna and Goldstein assessed the magnitude of this increase for two-dimensional ( $5 \times 5$ ) lattice proteins in which they used interaction energies derived from empirically measured data on amino acid interactions in real proteins (540). In the average sequence folding into a stable model structure, only 0.04–0.4% of single amino acid exchanges led to increased thermodynamic stability. In contrast, in sequences where mutational robustness had evolved in the way described, between 18 and 28% of mutations increased thermodynamic stability.

Again, such observations from model proteins, which suggest that selection can change mutational robustness and thermodynamic stability, have to be taken with a grain of salt. It is thus reassuring that empirical studies on real proteins are consistent with the evolvability of protein stability (60, 139). Specifically, experimental studies show that protein stability can vary genetically—a prerequisite for its evolvability—by manipulating one or a few amino acids. Examples include elimination of glycine residues or the introduction of proline residues into a protein. Both reduce a protein's flexibility and can thus increase its thermodynamic stability. Cases in point are a glycine to alanine substitution in the lysozyme of bacteriophage T4, which increases the enzyme's melting temperature by  $1^{\circ}\text{C}$ , and an alanine to proline substitution, which increase the enzyme's melting temperature by  $2^{\circ}\text{C}$  (ref. (60), chapter 17). Other experimentally proven ways to change protein stability include the introduction of cysteines that can form disulfide bridges, or the neutralization of the dipole formed by any  $\alpha$ -helix through suitably charged amino acids at either end of the helix (60). Random mutations also affect thermodynamic stability, but the extent to which they do so depends on the protein. For example, in proteins as different as myoglobin and T4 lysozyme, about one-quarter of mutations increase thermodynamic stability (540). This is not the case for the immunoglobulin G binding domain of a cell-wall protein of the bacterium *Peptostreptococcus magnus* (282). Most of 12 heavily mutated variants of this domain fold properly, but all of these variants are less thermodynamically stable than the wild-type. Such evidence indicates that some proteins are more thermodynamically stable than necessary for

proper folding, suggesting a role for natural selection in increasing thermodynamic stability beyond a necessary minimum.

Evolutionary differences in the stability of a well-studied class of proteins—enzymes—provide even stronger support for the notion that protein stability can change in evolution. Subtle conformational changes mediated by thermal noise are at the heart of an enzyme's ability to catalyze chemical reactions. If an enzyme's environment is too cold, the enzyme may move so slowly that catalysis does not occur at useful rates. If the environment is too hot, the enzyme's motions may be so drastic that its substrates have difficulty binding. Put differently, an enzyme's thermodynamic stability has an optimum that varies with environmental temperature. Concomitantly, key indicators of enzyme stability such as denaturation temperature and catalytic efficiency vary for organisms living at different temperatures. For instance, in crystallins—eye lens proteins often derived from metabolic enzymes—the temperature at which 50% of the protein's secondary structure gets lost depends on the organism's environmental temperature. Specifically, this denaturation temperature rises from 47°C for vertebrates adapted to a maximal environmental temperature of 0°C, to more than 55°C for vertebrates adapted to temperatures of more than 35°C (153). A similar pattern exists for the rate  $k_{cat}$  at which an enzyme converts substrate into product. For example, the  $k_{cat}$  of muscle-type lactate dehydrogenase at 0°C decreases by a factor five from organisms adapted to an average habitat temperature of 0°C to organisms adapted to an average temperature of 40°C (153).

It is mostly subtle sequence changes that cause such stability changes. Enzymes of the same function that occur in organisms adapted to increasingly high temperatures tend to contain more charged amino acids. Such amino acids can form strong ionic bonds with other amino acids, which stabilizes protein structure against the stronger thermal motions at higher temperatures. Enzymes adapted to high temperatures also tend to contain more amino acids with large side chains, which generally reduces the flexibility of a protein and thus increases its stability (153).

Thermodynamic stability can increase in the evolution of real proteins. But does natural selection favor *maximal* stability and its correlate, maximal mutational robustness? Put differently, are the proteins of a given structure that we observe in today's organisms more stable and robust than proteins folding into the same structure, but chosen at random in sequence space? We do not know, and the answer will depend on the protein. Maximal stability and robustness may be important for proteins whose primary role is to give structural support to a cell. However, it is detrimental to enzymes, because subtle conformational changes lie at the heart of their ability to catalyze chemical reactions. Not surprisingly, most enzymes are marginally stable, with a free energy difference between the

native and denatured state of 5–15 kcal/mol, the equivalent of a few hydrogen bonds (2–5 kcal/mol) (60).

## Missing Links

Many features of protein structures and their robustness are reminiscent of the results for RNA structures I discussed above. These include a discrepancy between rare and frequent structures, the higher average robustness of frequent structures, the ability to evolve robustness to amino acid changes, and the association between robustness and thermodynamic stability. Similar to well-studied RNA molecules, well-studied proteins—enzymes—need to be stable enough to fold into a well-defined structure, but not too stable to change their conformation for efficient catalysis. In other words, their evolution may favor optimal thermodynamic stability, which is not the same as maximal thermodynamic stability (and maximal robustness against mutations).

Despite such insights, many open questions remain about robustness in proteins. First, the nexus between thermodynamic stability and mutational robustness is not well studied in real proteins. Second, is the remarkable robustness of many real proteins an evolved feature? That is, are these proteins more robust than an average protein folding into the same structure, but chosen at random from sequence space? The fact that model proteins can evolve increased robustness argues for this possibility. The observation that many enzymes are marginally stable—and thus probably not maximally robust—casts doubt onto its generality. Finally, is structural innovation through point mutations as easy in proteins as it is for RNA? Recall that in RNA, most secondary structures occur around a small sequence neighborhood of any one RNA sequence. This means that one can reach many structures by relatively few mutations, and thus that large structural changes are easily achieved in RNA. Proteins may be different in this regard. Work on model proteins suggests that proteins of the same structure may be more clustered in sequence space than RNAs (53, 54, 335, 398). Evidence from real proteins supports this notion. For instance, enzymes with more than 40% sequence identity are very likely to catalyze the same chemical reaction (554). However, such evidence, although suggestive, is insufficient for a conclusive answer to this question.

# 6

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## Proteins and Recombination

What is robust? The structure and function of proteins.

What is it robust to? Recombination, that is, swapping of contiguous stretches of amino acids among proteins.

Recombination is a much more drastic genetic change than the point mutations in individual nucleotides and amino acids I have discussed thus far. It can replace multiple contiguous amino acids in a protein. On a larger scale, it can lead to complicated rearrangements of many genes. Are the chimeras that recombination creates true monsters, or are they often still well-functioning biological systems? In other words, how robust is protein structure and function to recombination? Unfortunately, compared to robustness to point mutations, the robustness of biological systems to recombination is little understood. However, because of recombination's importance, I will survey what little pertinent evidence there is. This evidence suggests that proteins may be surprisingly robust to recombination, and it suggests possible reasons for this robustness. One of them is the modular architecture of many proteins. After surveying the evidence, I will briefly discuss an important phenomenon related to recombination, lateral gene transfer, and how it relates to this book's central theme.

### The Importance of Recombination

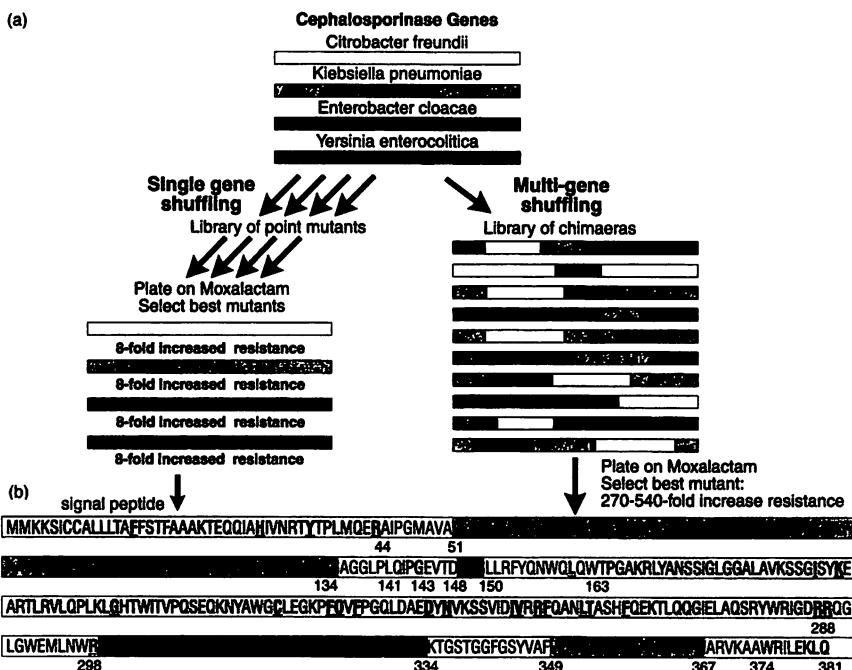
Recombination has been an important factor in protein and genome evolution. The following two observations offer the most convincing proof of this assertion on the protein level. First, the genomes of most organisms harbor thousands of proteins that consist of more than one domain. (A domain is a protein part with a characteristic amino acid sequence or tertiary structure that often folds autonomously (301).) Second, the same domain is often found in many proteins of very different functions. Taken together, these two observations indicate that many new proteins are generated by the shuffling of existing domains, and thus by recombination (190). The most prominent mechanisms to create new domain

arrangements move domains through transposable elements and rearrange them through unequal crossing over (336). These two mechanisms, as well as the more conventional reciprocal recombination of homologous DNA sequences, do not generally respect the boundaries of genes and the boundaries of domains within genes. That is, they can truncate and extend genes, as well as generate proteins that have suffered recombination within domains. Recombination thus has potentially serious consequences on protein structure.

It is important to be aware that the effects of recombination depend on the sequence similarity between the recombining genes. Assume that a recombination event occurs between two genes encoding proteins that differ by only 1% from an ancestral wild-type protein. If the two proteins have 100 amino acids, then the recombination product would differ in no more than one amino acid from them. Put differently, the effect of recombination on these proteins may be no more severe than that of single point mutations. The less similar two proteins are, however, the more drastic the effects of recombination on each of them. At first sight, one might think that recombining distantly related proteins must be extremely disruptive to protein structure and function, because it causes changes in multiple amino acids. However, both experimental and theoretical work indicates that this is not so. Recombination, even between distantly related proteins, may have surprisingly mild effects on protein function, and can even improve protein function.

## DNA Shuffling Experiments

An illustration of this observation comes from a technique that is used with great success to generate recombinant proteins in the laboratory (77, 92, 93, 329, 400, 514, 523, 644). In this technique, known as "DNA shuffling" (523), DNA sequences encoding variants of the same gene are pooled and then cut into small fragments using DNase I. This enzyme cleaves at random positions within a DNA molecule. In the resulting complex mixture of DNA fragments, many gene fragments overlap over short stretches of their DNA sequences. In the next step of the procedure, the individual double-stranded DNA fragments are denatured—separated into single strands—at high temperatures and reannealed afterward. The result are chimeric DNA molecules with short double-stranded regions that can serve as primers for DNA polymerase in a polymerase chain reaction. Multiple cycles of denaturation, reannealing, and synthesis of new DNA from the primed templates can yield recombinant genes of the same length as the "parental" genes. These recombined genes consist of multiple recombined fragments of the parental genes (523).



**Figure 6.1** (a) An experiment shuffling cephalosporinase genes from four microbial species whose names are indicated in the top panel of the figure. The right part of the figure illustrates the mosaic nature of recombinant proteins obtained by shuffling genes from all four species. The left part illustrates the outcome of four control experiments, each of which shuffled DNA from one of the four genes. The recombination products in these control experiments are identical to the parental sequence, except for point mutations introduced by the DNA polymerase used in the shuffling process. (b) The amino acid sequence of the most active chimera obtained in the DNA shuffling experiment. The segments derived from *Enterobacter* are shown in dark gray, those from *Klebsiella* in medium gray, and those from *Citrobacter* are shown in light gray. The white areas indicate where the recombination events took place. They cannot be unambiguously assigned to any one of the parental sequences. Amino acid point mutations are indicated by bold letters. The numbers at the beginning and end of each segment are coordinates of the respective amino acid residues from the genbank protein files of the wild-type enzymes. From (93).

An illustrative application of this technique comes from recombinant cephalosporinase genes. Cephalosporinases confer resistance against cephalosporins, a widely used class of antibiotics. In a single DNA shuffling experiment, Crameri and collaborators (93) recombined four cephalosporinase genes from distantly related microbes (Figure 6.1). The



**Figure 6.2** Predicted tertiary structure of the chimaeric cephalosporinase with elevated activity from Figure 6.1b. Different shades of gray indicate the different species origins of the amino acid sequences. The predicted tertiary structure of the  $\alpha$ -chain backbone has less than  $0.766\text{ \AA}$  root-mean-square deviation from the crystal structure of the *Enterobacter cloacae* wild-type enzyme. From (93) by permission of the Nature Publishing Group.

genes are 1.6 kilobases long and 58–82% identical at the DNA sequence level. Figures 6.1b and 6.2 show the amino acid sequence and structure of the most active chimeric product of this experiment. This chimeric protein is a patchwork of amino acid sequences derived from each of the four “parental” sequences. Relative to the best parental gene, it confers a 270-fold increase of resistance to the cephalosporin moxalactam. In four separate control experiments carried out under the same conditions, Crameri and collaborators subjected four homogeneous solutions of each of the four parental cephalosporinase genes to DNA shuffling. Because all the sequences in these control experiments are identical, their recombination products are also identical. The only variation among them occurs through replication errors of the DNA polymerase used in the shuffling protocol, that is, through point mutations. The best cephalosporinase gene emerging from these control experiments showed a mere 8-fold increase in moxalactam resistance (caused by point mutations) over the parental sequence (93). Thus, recombination among multiple distantly related genes may lead to a much greater improvement in enzyme activity than mere point mutations.

These experiments identify enzymes that confer increased antibiotic resistance among more than ten thousand products of DNA shuffling.

However, the fraction of recombination products that has no biological activity has not been determined. Thus, these experiments do not answer the question of how robust the function of any one parental sequence is to recombination. This question, however, has been addressed in two DNA shuffling studies. One such study used DNA shuffling to generate chimeras of human interleukin 12 (329). Interleukin 12 regulates the immune response to infectious diseases and can also inhibit tumor growth and abolish tumor metastases. It is a dimeric protein consisting of two different subunit polypeptides, p35 and p40, that interact with a receptor protein to carry out their biological function. In two separate DNA-shuffling experiments, one for the p35 and the other for the p40 subunits, Leong and collaborators (329) generated chimaeric subunits from human, rhesus monkeys, cows, pigs, cats, and dog interleukin 12 genes. Pairs of proteins from these organisms differed at between 8 and 65 out of more than 200 amino acids. Of the chimeras resulting from DNA shuffling, 80% contained DNA sequences from all ancestors. Strikingly, more than 95% of the p35 chimeras and 43% of the p40 chimeras retained the capability of interleukin 12 to induce the proliferation of human T cells (329).

Another pertinent study is a DNA-shuffling experiment of human  $\alpha$ -interferons (77).  $\alpha$ -Interferons can interfere with viral infections and can inhibit cell division. They thus have applications as antiviral and anticancer drugs. The human  $\alpha$ -interferons are encoded by more than 20 tandemly duplicated genes with 85–98% amino acid identity. Chang and collaborators used all these human genes in a DNA-shuffling experiment, and found that most chimeric interferons were biologically active (77). They analyzed in detail the DNA sequence and the biological activity of four randomly chosen chimeras. To do so, they used an assay that measures how strongly  $\alpha$ -interferons inhibit cell division in a human lymphoma cell line. The four chosen interferons differed at between 10 and 21 amino acids from the closest wild-type interferon. Yet all of these interferons were at least as active in the assay as the most active native human  $\alpha$ -interferon. If the fraction of active chimeras in the pool was very small, then it would be astronomically unlikely to find four randomly chosen chimaeras with such high biological activity.

### Why Robustness to Recombination?

Chang and collaborators (77) argue that protein activity may be robust to recombination because recombination among functional genes explores a tiny fraction of sequence space that is greatly enriched for functional sequences. To be precise, this fraction is tiny compared to all the possible

point mutation variants of the recombining proteins. A simple calculation (77) illustrates this idea. The members of the human interferon gene family vary at 76 amino acid positions, each of which harbors only a limited number of amino acids. Specifically, 57 positions harbor 2 different amino acids, 15 positions harbor 3 amino acids, and 4 positions harbor 4 amino acids. This amounts to a total of  $2^{57} \times 3^{15} \times 4^4 = 5 \times 10^{26}$  different possible recombination products for these genes. Contrast this with the possible number of point mutations at randomly chosen positions of these interferons. Any two  $\alpha$ -interferons differ at an average of 17 out of 166 amino acid positions. There are  $166!/(166 - 17)!(17!) = 7 \times 10^{22}$  possible ways to distribute 17 mutations over 166 positions. For any one of these ways, there would be  $20^{17}$  possible random mutants. Thus, in total, there would be  $7 \times 10^{22} \times 20^{17} = 9 \times 10^{44}$  random mutants of 17 residues, almost a factor  $10^{19}$  more than the possible  $5 \times 10^{26}$  recombination products. Thus, recombination among  $\alpha$ -interferon gene family members explores a tiny fraction (one  $10^{-19}$ th) of the sequence space accessible to random mutations. This population of shuffled chimeras, the argument goes, is highly enriched for functional sequences, compared to 17-step mutants of any wild-type interferon, most of which would have no biological activity (77).

This argument is plausible but it may be only one part of the explanation. In the above interleukin experiment, the DNA shuffling experiment involved genes from different mammalian species that shared a common ancestor many million years ago. This means that genes of these species do not recombine in the wild. In contrast, because  $\alpha$ -interferons are tandemly arranged in the same genome, they may continually recombine through unequal crossing over. The sequence variation in the human  $\alpha$ -interferons may thus be organized such that recombination events between them are minimally likely to disrupt gene function. In other words, robustness to recombination may be a response to repeated recombination events in these genes. Unfortunately, no available experimental evidence speaks to the question of whether exposure to frequent recombination events increases robustness to recombination.

### Insights from Computational Work

In the face of scarce empirical evidence, computational models of protein folding can provide some insights into the causes of robustness to recombination. Lattice protein models, which I introduced in chapter 5, have shown that recombination can greatly increase the extent to which mutational robustness to point mutations can evolve (635). However, they have not yet been used to ask whether robustness to recombination increases

after repeated exposure to recombination, and have instead focused on more modest goals. For instance, existing work shows that proteins are highly robust to recombination even without repeated exposure to it. This work, a study by Cui and collaborators (97) also suggests a probable mechanistic cause for such robustness, a cause experimentally confirmed in real proteins. Cui and collaborators analyzed lattice proteins consisting of hydrophobic and polar amino acids, where interactions between hydrophobic proteins are energetically favored (97). The investigators systematically analyzed all chains of 18 monomers that fold into a unique structure with a free energy smaller than alternative structures. They asked how reciprocal recombination among chains affects this structure. Despite obvious limitations—simple protein geometry, short chains, an oversimplified energy model, and stable folding as a proxy for protein function—this study yields basic insights into the robustness of proteins to recombination.

Some of the study's findings are reminiscent of results discussed in chapters 4 and 5: Among the 1475 different structures that the model proteins can adopt, there are many rare conformations. Specifically, 337 structures are adopted by only one sequence. In contrast, a few structures are very frequent, the most frequent of them being adopted by 4553 sequences. For more than 85% of the structures attained by more than one sequence, all encoding sequences can be connected through single (neutral) point mutations. The sequences encoding the remaining structures fall into up to four disconnected sets. The authors studied the effect of one-point reciprocal recombination events between two sequences that fold into a unique structure. Such recombination events essentially swap the first  $k$  amino acids of a model protein with the last  $(18 - k)$  amino acids of another protein. There are more than  $3 \times 10^8$  possible such recombination events between sequences that fold into a stable structure.

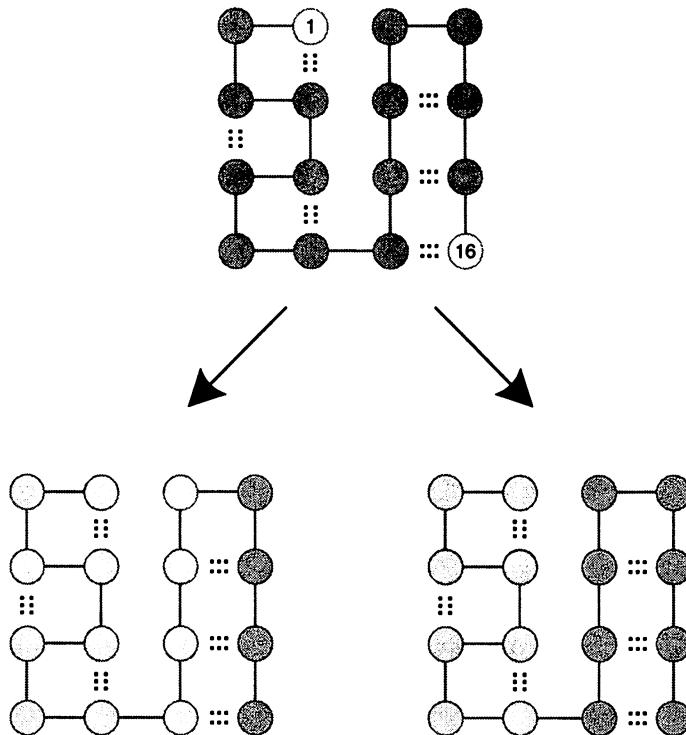
The authors' simple yet crucial finding is this. When pairs of sequences that fold into the same structure undergo recombination, 78.9% of recombination products fold stably into a structure and the vast majority of them (99.3%) adopt a structure identical to that of the parents. This number suggests striking robustness of proteins to recombination, especially if one considers that only 2.4% of model proteins attain any stable structure at all. Thus, most recombination products remain not only within the very small part of sequence space folding into some stable structure, but also within the even smaller part folding into the same structure as their parents. Because the analysis is based on a systematic exploration of sequence space, the observed robustness has not evolved in response to repeated recombination, but is a generic feature of these model proteins.

What are the mechanistic causes of such robustness? To answer this question, it is important to bear in mind that recombination exchanges

contiguous stretches of amino acids. That is, amino acids near each other in a parent are much more likely to co-occur in a recombination product than amino acids far apart from each other. This observation lends itself to an explanation for robustness, an explanation that rests on two premises. First, the disruptive effects of recombination depend on the number of disrupted amino acid interactions in the folded protein. The more amino acid interactions a recombination event disrupts, the more drastically a protein's structure will be affected. Second, if many amino acid interactions are local, that is, if they involve amino acids near each other on the amino acid chain, then recombination—swapping of adjacent amino acids—will preserve more such interactions than exchange of noncontiguous amino acids. Put differently, recombination will tend to leave a protein's structure intact, if local interactions are more important than global interactions in determining protein structure (Figure 6.3).

The first assumption, that recombination is more disruptive if it disrupts many amino acid interactions, holds for real proteins, as shown by Voigt and collaborators (569). These investigators used experimentally characterized protein structures to determine the number of disrupted amino acid interactions in reciprocal recombination events at different positions of a protein. For several proteins, including cephalosporinase, subtilisin (a protease important in the detergent industry), and cytochrome P450 (an enzyme that can oxidatively detoxify hydrophobic chemicals), these authors calculated amino acid positions where recombination disrupted a minimal number of interactions. Strikingly, these minimally disruptive recombination positions coincide with positions at which recombination preserves protein function or structure in laboratory experiments. Many of these positions also correspond to the boundaries between recognizable secondary structure elements, such as an  $\alpha$ -helix followed by a  $\beta$ -strand, or two  $\beta$ -strands connected by a hairpin turn. Exceptions to this principle exist and can be instructive. One example regards recombination within loops, which are often considered mere linkers between more important parts of the protein. Nonetheless, recombination between loop regions can sometimes be highly disruptive. In such cases, a loop often connects two secondary structure elements—say, an  $\alpha$ -helix and a  $\beta$ -strand—which are linked by many amino acid interactions.

The occurrence of minimally disruptive recombination events at the boundaries of secondary structure elements—or combinations thereof—also supports the importance of local interactions in determining protein structure. In fact, secondary structure elements are defined by specific patterns of local amino acid interactions. As a parenthetical note, the number of amino acids in one protein that are effectively changed ("mutated") as a result of a recombination event does not correlate well with the number of disrupted amino acid interactions. In other words, a recombination



**Figure 6.3** Robustness to recombination and local amino acid interactions. Illustration of the effects of recombination on amino acid interactions necessary for stable folding. Shown is a simple lattice model protein consisting of 16 amino acids, represented as black dots. Black lines represent peptide bonds, and dotted lines correspond to noncovalent interactions between amino acids. Notice that interactions occur only among amino acids 1 through 8 and among amino acids 9 through 16, but not between these two groups of amino acids. This means that local interactions contribute more to this model protein's structure than interactions among distant amino acids. This has consequences for the effects of recombination. For example, a recombination event between amino acids 8 and 9 of two proteins folding into this structure will not disrupt any interactions (lower right), whereas recombination between amino acids 12 and 13 may disrupt up to three interactions (lower left). From Figure 1 in (569).

event may change many amino acids but disrupt few interactions and vice versa (569).

In sum, a limited amount of experimental work shows that proteins can tolerate many and drastic recombination events. A number of random mutations equal to the number of amino acids changed in such recombination events would generally have more severe detrimental effects. Part

of the reason is that local interactions among nearby amino acids are important in determining protein structure. Some proteins, especially members of closely linked gene families may experience continual recombination events while under pressure to maintain their function. Whether such proteins have evolved increased robustness to recombination is unknown.

### An Aside on Lateral Gene Transfer

Thus far, I have focused on the effects of recombination on individual proteins. However, recombination can also change biological systems on a much larger scale. It can potentially recombine multigene systems such as metabolic pathways or genetic networks to create systems with new structure and function. Laboratory evidence for recombination's power comes again from DNA-shuffling experiments, where not only individual genes but multigene systems or entire genomes can be successfully recombined (92, 644). Such experiments can generate rapidly—more rapidly than point mutations—bacterial strains with new and desirable features. These features include antibiotic production and tolerance against environmental toxins (92, 644). Unfortunately, none of the available experiments asks how robust genetic systems beyond proteins are robust to recombination. That is, we do not know what fraction of recombined systems preserve their original function, acquire a new function, or disintegrate altogether. Only the successful outcomes of recombination events are seen.

Human experiments are, as usual, eclipsed by nature's ongoing grand experiment. For recombination on or above the gene level, this is illustrated by the abundance of lateral gene transfer, a kind of recombination event that adds new genes to a genome. Lateral or horizontal gene transfer is the transfer of genes into a host organism from a donor organism that is not the host's immediate ancestor. The incidence of lateral gene transfer in eukaryotes is unknown, but it is pervasive in prokaryotes: Of the order of 10% of genes in a typical prokaryotic genome may be the result of recent horizontal transfer (407). The donor organism need not even be closely related to the host: Gene transfer may involve not only bacteria of different species, but also of different classes or phyla. It may even involve prokaryotes of different domains, such as bacteria and archaea (57). The genes transferred can endow a host genome with biological features critical for survival. Sometimes, as in the case of antibiotic resistance traits, the transferred features result from the action of only one gene. In other cases, whole suites of genes are transferred.

One prominent example of a horizontally transferred multigenic trait is the ability to fix nitrogen. Nitrogen fixation is the transformation of molecular nitrogen,  $N_2$  to ammonium,  $NH_4^+$ . It is important to all plant

life and is carried out only by prokaryotes. Nitrogen fixation is accomplished through a multiprotein complex called the nitrogenase complex, of which different variants exist. Horizontal transfer of genes for the nitrogenase complex has occurred among prokaryotes of all levels of evolutionary separation, such as between bacterial genera, classes, and phyla (57). Another example of an important horizontally transferred gene is tetrapyrrole-based photosynthesis, photosynthesis based on chlorophyll and its relatives (57). In some cases, such as the distantly related  $\alpha$ -proteobacteria and  $\beta$ -proteobacteria, more than two dozen genes required for photosynthesis may have been jointly transferred (250). Further examples include suites of genes responsible for aerobic respiration, the biosynthesis of isoprenoids (important membrane components) and the ability to survive in extreme environments. Transfers of large groups of genes are often facilitated by the clustering of genes in the genome, and by the co-occurrence of such genes on easily transferred plasmids (57, 323).

Lateral gene transfer is a peculiar kind of recombination event, where the host genome is typically left intact except for the addition of new genes. Thus, lateral gene transfer may be less disruptive than the kind of recombination events used in DNA-shuffling experiments. However, beyond this speculation, we do not know how often lateral transfer of one or more genes is successful. That is, we do not know how robust biological systems are to this transfer of genes. As in many laboratory evolution experiments, we simply see successful outcomes, but there may be countless casualties behind these successes. Why, then, discuss horizontal gene transfer here? First, because it raises the question of how often lateral gene transfer succeeds, how robust organisms are to it. Second, because it is a phenomenon too important to ignore when discussing recombination. Third, when viewed in the context of recombination, horizontal gene transfer points to a possible relation between robustness and the phenomenon of modularity in biological systems, which I now briefly address.

### Recombination, Robustness, and Modularity

Robustness is only one of several features of a biological system that affect its ability to evolve. (I will say more about definitions of evolvability and its relation to robustness in chapter 14.) Another such feature is modularity (224, 287, 570). Modularity occurs on all levels of biological organization. Its meaning is best illustrated with examples. The parts of an enzyme responsible for its catalytic activity and for the regulation of this activity can sometimes be manipulated separately through different amino acid changes (570). In this sense, catalytic activity and regulation

are modular aspects of an enzyme's function. One and the same genetic network is sometimes deployed at different times, in different places, in different organisms, and to different effect (183). Such a network can be viewed as a module whose different functions emerge from the different contexts in which it is deployed. On an even higher level of organization, eukaryotes and their endosymbiotic organelles provide perhaps the most striking example. Once free-living organisms, these organelles now serve as cellular modules responsible for respiration (mitochondria) and photosynthesis (chloroplasts). The central topic of this chapter, the generation of new protein functions through recombination of domains, as well as evolution through lateral gene transfer, is only a further example of how evolutionary innovation becomes possible through modularity.

An adequate discussion of modularity and its relation to evolutionary innovation would fill another book. Here I only want to raise one question: Are modular systems also robust to mutations? Human engineers favor modular system designs. Part of the reason is that a flaw in one system module affects only one aspect of the system's function and can thus be easily identified and fixed. In analogy, it could be argued that modularity provides mutational robustness to organisms, because mutations in an individual module, be it a protein or a network, cause a defect of limited extent. However, it takes little to find the loophole in this argument: Even though a mutation may affect only one module of a biological system, the organism will still be eliminated if this module is essential for survival or reproduction. One might thus conclude that modularity and robustness have little to do with each other. However, some of the material on recombination I discussed above suggests a link between robustness and modularity. Proteins are robust to recombination, especially when this recombination occurs between regions that one might call modules, regions within which many amino acids interact. One might thus think that the modular structure of proteins is at the heart of protein robustness to recombination. Similarly, complex traits such as aerobic respiration and nitrogen fixation are modular traits, because discrete groups of genes facilitate them. If the ease of transferring such genes indicates robustness of organisms to such transfer, then modularity and robustness may be intimately linked. This tentative link of robustness and modularity is, however, unexplored beyond such anecdotal examples.



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## **Part II**

### **ROBUSTNESS ABOVE THE GENE LEVEL**



## Regulatory DNA Regions and Their Reorganization in Evolution

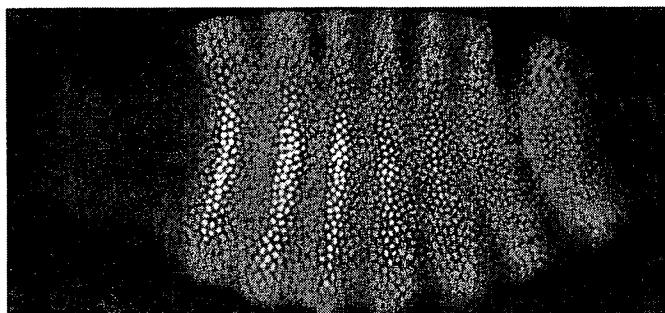
What is robust? A gene's expression pattern.

What is it robust to? Drastic changes in the gene's regulatory regions caused by mutations.

As I discussed in chapter 1, two main approaches can provide information on a biological system's robustness. The first consists of many experimental perturbations of the system's parts. An example is the generation of thousands of amino acid changes in a protein. The second consists of comparing systems in related species, systems that derive from a common ancestor and that represent different solutions to the same biological problem. An example is the comparison of proteins with similar function and a common ancestor in widely divergent species. Such a comparison can show that alternative—sometimes drastically different—solutions to a biological problem exist. The existence of such alternatives also implies that the different parts of a “neutral space” represented by these solutions are connected through a series of mutations that allow an evolutionary transition from one part of this space into another. This second line of evidence is much more indirect and less conclusive. However, it is the only line of evidence that is available for many systems. Part of the reason is that systematic perturbations can be difficult to come by, either because a system's structure is poorly understood or because of technological limitations. The main examples from this chapter are of this second kind, where robustness is inferred from the outcome of biological evolution. The biological problem here is how to assure proper expression of a gene in space and time. This problem can be solved in very different ways, through regulatory regions that have different organization and that are bound by different suites of transcriptional regulators.

### A Case Study of Enhancer Evolution

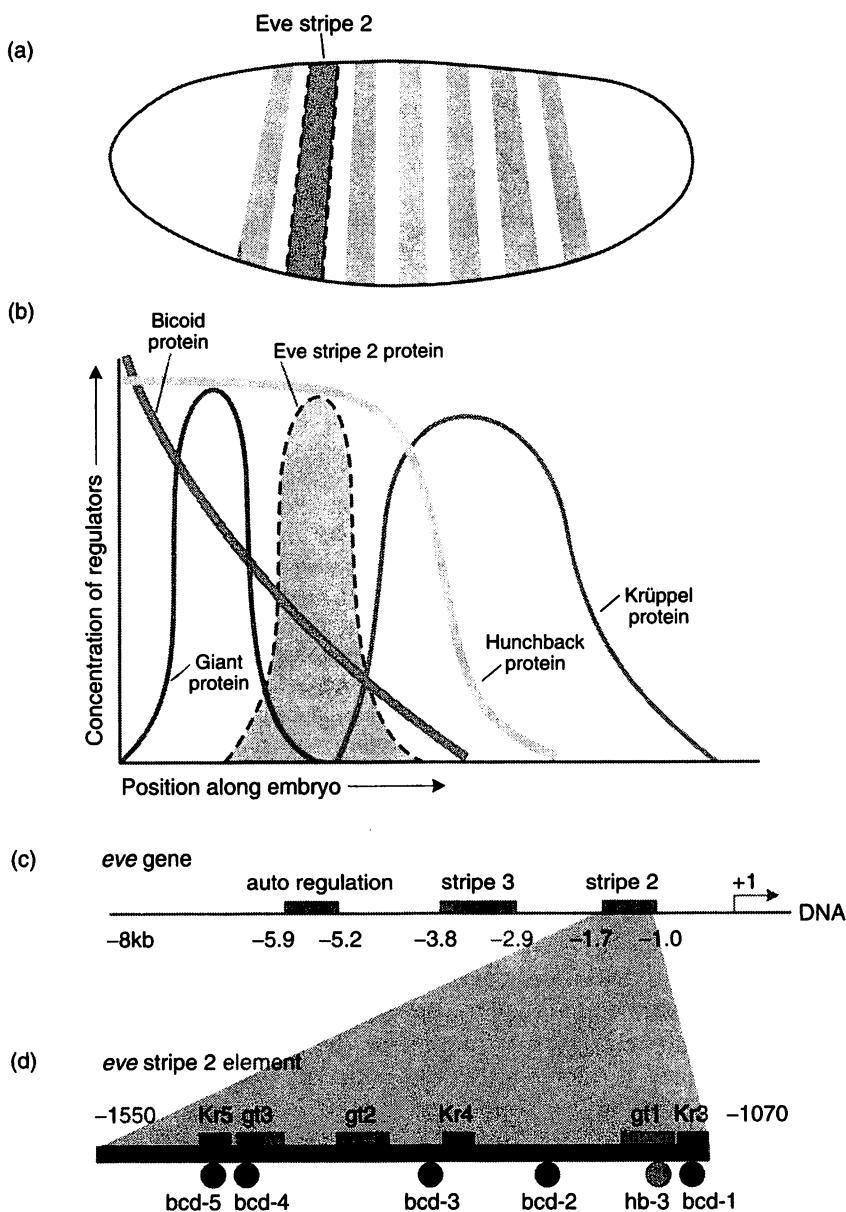
The system at issue is a gene, together with its regulatory region (enhancer), which may be bound by dozens of transcriptional regulators.



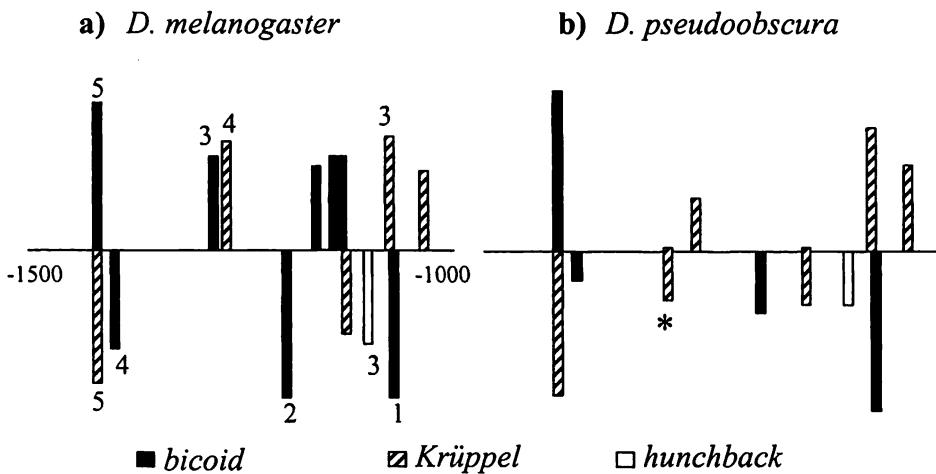
**Figure 7.1** Expression of *even skipped* in seven narrow stripes of a *D. melanogaster* embryo 2–3 hours after fertilization. Image obtained from the FlyEx database (<http://flyex.ams.sunysb.edu/>; image mat1.eve) by permission of David Kosman and John Reinitz.

Jointly, these transcriptional regulators ensure that the gene is expressed at the right time and location to allow processes such as embryonic development to proceed properly, thus ensuring survival and reproduction of the organism. I begin with a well-studied example, that of the *even-skipped* (*eve*) gene of the fruit fly *Drosophila melanogaster*. This gene is essential for proper fly development. Specifically, *eve* is required for the subdivision of the early *Drosophila* embryo into the regions that will eventually become the fly's segments, a process which I discuss further in chapter 10. *Eve* is expressed in seven narrow—three to four cells wide—and evenly spaced stripes (Figure 7.1) beginning approximately two hours after fertilization. This pattern of gene expression is necessary for subdividing the embryo into the multiple segments that are among the defining features of insects. Abolishing *eve* expression leads to a loss of about half the embryo's segments—a lethal mutation. Expression of the seven *eve* stripes is driven by separate pieces of regulatory DNA, separate stripe-specific enhancer regions. The best analyzed among these stripe-specific enhancer regions is the enhancer responsible for expression of the anterior stripe 2 (Figure 7.2). This *eve* stripe 2 enhancer is complex, consisting of multiple binding sites for four different transcriptional regulators. Two of these transcriptional regulators, Bicoid and Hunchback, activate *eve* expression, whereas two others, Giant and Krüppel, repress it (72). The four regulators have themselves well-defined expression patterns (Figure 7.2), which are jointly responsible for the precise expression of *eve* in the narrow stripe 2. (The other six stripes of *eve* expression are driven by different combinations of transcriptional regulators.)

The DNA sequence of the *eve* stripe 2 enhancer has been determined not only for *Drosophila melanogaster* but also for a number of other species



**Figure 7.2** (a) *Eve* stripe 2; (b) its regulation by four transcriptional regulators—Bicoid, Hunchback, Gap, and Krüppel; (c) part of *eve*'s regulatory region; (d) *eve*'s stripe 2 enhancer. From Figure 3.7. in (72).



**Figure 7.3** *Eve* stripe 2 enhancer organization in (a) *D. melanogaster* and (b) *D. pseudoobscura* with respect to binding sites for transcription factors Bicoid, Krüppel, and Hunchback (343). The height of each bar corresponds to the position-weight matrix score (39, 152, 599) of a binding site, which is correlated with the affinity of a transcription factor to its site. Binding sites numbered in (a) represent experimentally verified functional binding sites. Bars above and below the main axes correspond to binding sites that are encoded on the top and bottom strand of DNA, respectively. Functional importance of the rightmost (unlabeled) Hunchback site is indicated by its conservation across species. Multiple differences relative to (a) are visible in (b), including the disappearance of site 3 for Bicoid, the disappearance of the cluster of three Bicoid sites in the right half of (a), the appearance of a new site for Krüppel binding (\*), and the weakening of the position-weight matrix score of multiple other binding sites other sites. Redrawn from Figure 1dg in (343).

within the genus *Drosophila*. It varies substantially among these species: Among 13 species within the *Drosophila* species group, none of the transcription factor binding sites found in the *Drosophila melanogaster* stripe 2 enhancer are completely conserved (313, 342–344). The most detailed functional comparison of *eve* stripe 2 enhancers is available for *D. melanogaster* and *D. pseudoobscura*, two species that shared a common ancestor 40–60 million years ago (46). Figure 7.3 shows the organization of their respective stripe 2 enhancers, which shows a number of significant differences among the two species. The first of these differences is that one of the binding sites for Bicoid (the black bar labeled “3” in Figure 7.3a) is absent in *D. pseudoobscura*, although this site is essential for proper regulation of *eve* in *D. melanogaster* (502). Based on its pattern of occurrence in other species, this binding site may be a new acquisition of

*D. melanogaster* (342). Second, the *D. pseudoobscura* stripe 2 enhancer has an additional binding site for the transcription factor Krüppel (labeled with an asterisk in Figure 7.3b). Thirdly, the *D. pseudoobscura* binding sites Bicoid-4, Bicoid-2, Krüppel-4, and Hunchback-3 (Figure 7.3) probably bind their respective transcription factors much more weakly than the corresponding sites in *D. melanogaster*. This assessment is based on a comparison of their DNA sequences to multiple binding sites whose affinity has been empirically validated. (The degree to which a binding site matches other binding sites can be encapsulated in a “position weight matrix” score that is demonstrably correlated with binding affinity (39, 152, 599).) Fourthly, as observed in other *Drosophila* species, the spacing between binding sites in the *eve* stripe 2 enhancer varies widely between *D. melanogaster* and *D. pseudoobscura*.

### Changes in the Enhancer Make a Difference

Despite these substantial changes, the *D. pseudoobscura* stripe 2 enhancer functions perfectly in *D. melanogaster*. That is, when placed upstream of a gene whose expression is easily monitored, it drives the expression of the gene with the same temporal and spatial pattern as the native *D. melanogaster* stripe 2 enhancer. This shows that the function of the enhancer has been preserved over millions of years of evolution and it suggests that *eve* is under strong selection to maintain its expression pattern. It also raises the possibility that none of the changes that occurred in the *D. pseudoobscura* promoter are functionally significant. That is, perhaps neither the changed binding sites nor their spacing was important for the function of the *eve* stripe 2 enhancer. However, several lines of evidence argue against this possibility.

First, as I mentioned above, the binding site Bicoid-3, which is absent in *D. pseudoobscura*, is required for proper regulation of *D. melanogaster eve* expression (502). A second, drastic demonstration that some of the evolutionary changes affect enhancer function comes from chimaeric *eve* stripe 2 enhancers. Ludwig and collaborators tested the function of two such enhancers in *D. melanogaster* (343). One chimaeric enhancer contained the left (proximal) part of the *D. melanogaster* stripe 2 enhancer, but the right (distal) part of the *D. pseudoobscura* enhancer. The other contained the proximal part of the *D. melanogaster* enhancer, but the distal part of the *D. pseudoobscura* enhancer. In both enhancers, the breakpoint occurred less than 20 nucleotides upstream of the bicoid-3 site in *D. melanogaster* (Figure 7.3). Unlike the wild-type enhancers from either species, these chimaeras did not confer proper gene expression in *D. melanogaster*. Both of them showed a widening of stripe 2 expression,

and in one of them the stripe was shifted toward the posterior end of the embryo (343).

A third line of evidence is more indirect. It comes from patterns of molecular evolution in the multiple *Drosophila* species where the stripe 2 enhancer was studied (342, 343). Although no one transcription factor binding site is completely conserved, most sites underwent no more than one nucleotide change relative to the *D. melanogaster* site. The *eve* stripe 2 enhancer and other enhancers of *Drosophila* developmental genes evolve much more slowly than DNA regions that are not subject to any constraints on their DNA sequence. This indicates that not just any nucleotide substitutions are allowed in their transcription factor binding sites, that many mutations affecting properties of this enhancer were eliminated from the evolutionary record, and thus that evolution of this enhancer is constrained.

Taking this evidence together, the following picture emerges. The *D. melanogaster* and *D. pseudoobscura* *eve* stripe 2 enhancers have functionally different organizations. However, these organizations achieve the same goal, proper expression of the *even-skipped* gene during development. They are found in two lineages related by common ancestry, which indicates that these different organizations can be reached from one another through a continuous string of mutational changes that does not affect *eve* gene regulation.

The example also speaks to the context-dependence of mutational effects. Although the Bicoid-3 binding site is essential in *D. melanogaster*, it does not even exist in *D. pseudoobscura*. The evolutionary changes that can be tolerated in one part of the enhancer must depend strongly on the changes that occurred elsewhere during the enhancer's evolution. Unfortunately, no evidence speaks to the question of whether the string of changes that allowed the transition between enhancer organizations were slightly beneficial or deleterious—and thus effectively neutral—or truly neutral.

### The Sea Urchin *Endo 16* Gene

Unfortunately, despite a large literature on transcriptional regulation and its evolution (628), examples of enhancers whose evolution has been studied at this level of detail are few and far between. Other available studies are akin to puzzles with important missing pieces. However, they can still provide tentative support for the basic tenet that regulatory regions with quite different organization can drive one and the same gene expression pattern (96, 110–112, 354, 355, 440, 465, 482, 623).

A case in point is a study on the expression of the gene *Endo16* in two related sea urchin species, *Strongylocentrotus purpuratus* and *Lytachinus*

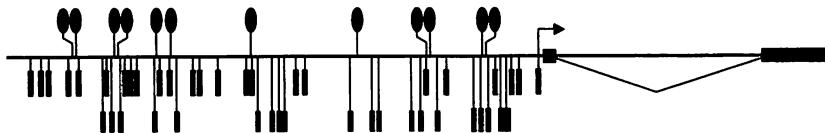
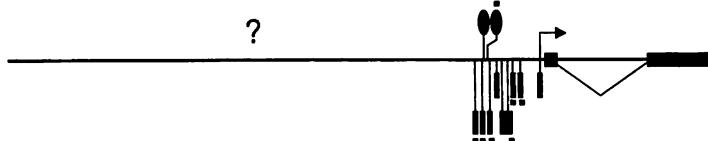
(a) *Strongylocentrotus purpuratus*(b) *Lytechinus variegatus*

**Figure 7.4** Time course of *Endo16* gene expression in two sea urchin species, *Strongylocentrotus purpuratus* and *Lytechinus variegatus*. From left to right, the panels show a temporal progression from the blastula stage of embryogenesis through gastrulation, to the pluteus larva, a life-stage characteristic of indirect developing sea urchins. The embryonic regions where *Endo16* is expressed are shown in black. The expression patterns of *Endo16* in the two species are very similar, except for one region that is to become the hindgut (indicated by the arrow pointing to the left). Specifically, *Endo16* continues to be expressed in the hindgut in *L. variegatus* but not in *S. purpuratus*. From Figure 3 in (465).

*variegatus*. These species diverged more than 30 million years ago. Their *Endo16* gene encodes a large extracellular protein that may play a role in cell adhesion. *Endo16* is expressed in a spatially restricted pattern during embryogenesis, a pattern that is very similar in the two species (Figure 7.4). However, despite this similarity in expression, most of the similarity in the regulatory regions driving this expression pattern has eroded beyond recognition.

The expression of the *Endo16* gene in *S. purpuratus* is driven by a 2200-base-pair-long regulatory region of extremely complex architecture (638, 639) (Figure 7.5). Specifically, this regulatory region contains some 56 binding sites for more than a dozen different transcriptional regulators. These binding sites can be arranged into seven adjacent groups or modules that serve multiple different purposes. For example, module A (not labeled in the figure) consists of the 10 transcription factor binding sites closest to the gene's transcriptional start site. It drives expression of the *Endo16* gene in the blastula stage of development and in the developing gut. Module B, consists of seven adjacent transcription factor binding sites and is responsible for maintaining *Endo16* expression in the gut of the larva. Other modules are responsible for repressing *Endo16* expression in some body regions or enhancing *Endo16* expression further in the expressed regions.

In *L. variegatus*, a regulatory region of similar length as that in *S. purpuratus* is sufficient for correct *Endo16* expression during embryonic

(a) *Strongylocentrotus purpuratus*(b) *Lytechinus variegatus*

**Figure 7.5** Regulatory regions driving *Endo16* gene expression in *S. purpuratus* and *L. variegatus*. (a) The regulatory region of *S. purpuratus* *Endo16* has a complex architecture with 56 binding sites for different transcription factors (638, 639). Large ovals indicate 12 binding sites for 12 unique transcription factors, each of which binds only one site within the regulatory region. Rectangles connected to the regulatory region via long lines indicate binding sites for a total of six transcription factors, each of which has two or more binding sites in the regulatory regions. Rectangles connected to the regulatory region via short lines indicate 23 binding sites for the structural protein GCF1, which stabilizes DNA looping. All these binding sites can be grouped into seven different “modules” (not shown) according to their function in regulating *Endo16* gene expression (638, 639). (b) The *Endo16* regulatory region of *L. variegatus* (465). Only a small-group of transcription factor binding sites has been conserved relative to *S. purpuratus*. A small square indicates that a nucleotide substitution, insertion, or deletion occurred in an *L. variegatus* binding site relative to *S. purpuratus*. After Figure 7 in (465).

development (Figure 7.5). Strikingly, however, the two regulatory regions are very dissimilar in sequence. Only the 10 transcription factor binding sites closest to the transcription start of *Endo16*—corresponding to module A—are conserved. They have suffered multiple mutations since the divergence of the two species, mutations whose functional significance is unclear. None of the remaining—more than 40—transcription factor binding sites exist in the *L. variegatus* regulatory region. In addition, the extent of nucleotide differences in the completely diverged part of the regulatory regions is similar to that in an intron that is not involved in transcriptional regulation. It is thus possible that most of the regulatory region may evolve neutrally. That is, natural selection may not constrain or favor nucleotide changes in it. Despite such nearly complete sequence divergence, the two regulatory regions drive gene expression in very similar ways.

Is it possible that the conserved transcription factor binding sites are sufficient for driving *Endo16* expression? Yes, but this is perhaps unlikely. The reason is that these transcription factor binding sites are insufficient to drive such gene expression in *S. purpuratus*. Is it possible that binding sites with the same activity as the *S. purpuratus* sites occur in the completely diverged regions? Yes, but most transcription factors bind to well-defined sites. Although some sequence variation in binding sites is permissible, the complete absence of variants resembling functional sites makes this unlikely. It may well be that a different, unrecognized set of transcriptional regulators bind the *L. variegatus* upstream regulatory region.

Additional evidence indicates that divergence in regulation has not been restricted to the transcriptional regulation regions. For example, when the *S. purpuratus* regulatory region is placed into *L. variegatus*, it drives expression in embryonic regions where *Endo16* is not normally expressed in either species. If the transcriptional regulators and their activity in the two species had been preserved, one would expect that this regulatory region would drive expression exactly as in its “native” species. Its inability to do so means that some transcriptional regulators interacting with it have diverged in their activity or expression. In sum, despite many uncertainties in how this regulatory region has diverged, the evidence indicates that two very similar gene expression patterns during sea urchin development can be established by different (perhaps fundamentally different) means in two closely related species.

### Further Relevant Case Studies

Other examples in a similar vein include the *unc-119* gene from the nematode *Caenorhabditis elegans* (354). Of unknown biochemical function, this gene is essential for proper nervous system functioning. The nematode *Caenorhabditis briggsae*, which shared a common ancestor with *C. elegans* 20–50 million years ago, contains a gene with 90% amino acid conservation to *unc-119* of *C. elegans* (354, 355). Despite this high conservation, which indicates strong functional constraints, introns and regulatory regions of *unc-119* are highly diverged between the two species. In more than 400 base pairs upstream of the translation start, less than 80 nucleotides are conserved. These conserved stretches occur in three regions that contain putative binding sites for transcription factor binding sites, but their spacing varies widely. Two of these regions overlap in *C. briggsae* but not in *C. elegans*, and one of them overlaps with the protein coding sequence in *C. elegans*, raising the possibility that it might not regulate gene expression at all in this species. Despite these

hints at functional differences, both regulatory regions drive gene expression identically in *C. elegans* (354, 355).

In a larger scale study that speaks to the question of variable enhancer organization, Dermitzakis and Clark (112) compared well-characterized regulatory regions of 51 human genes to their rodent counterparts. They included well-characterized genes of highly conserved function such as those encoding myoglobin, oxytocin, and preproinsulin. Between 32 and 40% of transcription factor binding sites that are functional in humans are not functional in rodents. This suggests a high turnover of transcription factor binding sites in the 70–100 million years since the human and rodent lineages diverged. A potential caveat to this high turnover rate is that some of the analyzed genes may have diverged in expression pattern, although the use of conserved genes with similar function in this study makes this less likely.

This potential caveat has been alleviated in a more recent study (110), which analyzed the evolution of several enhancers of developmental genes with conserved functions within the *Drosophila* species group. The study used a combination of DNA sequence analysis and computational prediction of transcription factor binding sites. It showed that the enhancers can undergo substantial reorganization through the appearance and disappearance of binding sites for transcriptional regulators. The study also shows that many fewer mutations are preserved in the evolutionary record than actually occurred in these enhancers. This implies that many mutations really affect the function of individual binding sites for transcriptional regulators.

The importance of regulatory DNA regions in evolution has long been recognized (286). Even stretches of DNA very distant from a gene's coding region can influence its expression via transcription factor binding sites that can be as short as six base pairs (6 bp). Such binding sites can appear and disappear rapidly through point mutations, and regulatory regions thus are deep reservoirs of variation that can profoundly affect evolution. Stone and Wray (527) asked how long it would take for new transcription factor binding sites of 6-bp length to appear in short stretches of DNA (2000 base pairs) through random point mutations, and how long it would take for such sites to become fixed in a population by random genetic drift alone. For *D. melanogaster* this expected fixation time is a mere 24 years. A regulatory region in *Drosophila* may of course be many times larger than 2000 base pairs, and natural selection may also play a role in driving newly evolved binding sites to fixation. Thus, evolution of new binding sites, and concomitant change of promoter organization can occur on very short time scales. Supporting this notion are the results of laboratory evolution experiments in microbes. During such

experiments genetic change in the expression of thousands of genes occurs within a mere few hundred generations (151).

Taken together, all these studies suggest that functionally different alternative promoter organizations that drive the same gene expression pattern not only exist, but can also emerge rapidly in evolution. Unfortunately, there are few thoroughly studied examples of evolving regulatory regions, examples where the functional significance of changes in transcription factor binding is known. Furthermore, studies comparing promoter architecture in *many* closely related species, which would provide more conclusive evidence, are completely absent. Finally, most existing work does not focus on genes with conserved expression—as is necessary to study robustness—but on genes with diverged expression in two or few species (72, 98, 170, 302, 435, 495, 529, 533).

What final picture on the robustness of regulatory regions will emerge once the evolution of many promoters has been thoroughly characterized? I surmise that the situation will be similar to that of proteins with their skewed distribution of structures (chapter 5). Recall that a few protein structures show little robustness, whereas most others can accommodate radical amino acid changes. The sequences folding into robust protein structures form a large part of sequence space and are connected through individual point mutations. Analogously, some regulatory regions may have a rigid organization, with little robustness to mutation, and a conserved organization on an evolutionary time scale. However, the majority of regulatory regions can tolerate radical reorganizations that leave their biological properties intact. I argue later (chapter 13) that robust biological systems occupy a larger fraction of the neutral space of alternative solutions to a biological problem. This alone would make them easier to find through the blindly groping search typical of biological evolution. For this reason, the majority of regulatory regions may turn out to be very robust to mutations. If so, the paramount question becomes: what architectural features endow a regulatory region with robustness?

# 8

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## Metabolic Pathways

What is robust? The flux of matter through a metabolic pathway and the pathway's output.

What is it robust to? Drastic changes in enzyme activity.

In diploid organisms, null mutations—mutations in which a gene loses its function—at one out of its two alleles often have no or little phenotypic effect. This observation encapsulates the phenomenon of dominance. Dominance is the facet of genetic robustness that first received major attention in the literature. This chapter discusses the origin and evolution of dominance in metabolic systems, where the mechanistic basis of dominance is especially well understood. However, the material in the chapter also speaks to robustness in metabolic systems beyond the phenomenon of dominance.

In the most general terms, dominance means that a phenotypic feature of an organism is robust to changes in gene dosage of 50%, or to a corresponding 50% change in the concentration of a gene product. In this sense, it is the wild-type allele (+) that is dominant (in its phenotypic effect) over a null-allele (-), because if both alleles occur together (+/-), this heterozygote's phenotype is wild-type (+). Fisher (155, 156) proposed the first evolutionary explanation of this phenomenon. According to him, dominance is an evolved adaptation of genetic systems to mutations. Fisher postulated the existence of modifier genes different from the gene at which dominance is observed. The function of a modifier gene is to modify the phenotypic effect of a heterozygote (+/-) at this “primary” gene, a modification that reduces any negative fitness effect a heterozygote suffers. According to this idea, a heterozygote phenotype is intermediate between the two homozygote phenotypes (+/+, -/-) when a null allele first arises. Dominance subsequently evolves through selection of alleles at the modifier gene that completely suppress the effects of the null allele.

It was not long after its publication that a major controversy arose around this model. In this controversy, Sewall Wright was Fisher's most prominent opponent (633). Fisher's model encounters two main criticisms. The first of them regards the boundary conditions, such as selection

coefficients and population sizes necessary for the evolution of dominance. Selection on a dominance modifier, the argument goes, may be too weak to account for the many genes with dominant alleles in a typical genome. (Chapter 16 discusses the reasons in the more general context of evolved genetic robustness.) A second criticism is that the model is unconcerned with the mechanistic or physiological basis of dominance and its modification. There may simply be no genes whose purpose is to modify dominance at other genes, and dominance may best be understood as a by-product of interactions among genes with important biological functions (79, 94).

Taken together, these criticisms proved fatal for Fisher's model. Over time, mechanistic explanations of dominance emerged that do not require dominance modifiers (267, 415, 481). The subject of the present chapter is an especially simple yet elegant mechanistic explanation of dominance first proposed by Kacser and Burns (266, 267). This explanation regards dominance in genes for metabolic enzymes, enzymes necessary for biosynthesis and energy production. To be sure, the mechanistic explanation of dominance for other genes—transcriptional regulators, transport proteins, cytoskeletal components—may be very different. However, a considerable fraction of a genome is devoted to metabolism—some 20% for free-living microbes such as yeast (371)—which implies that this class of models applies to hundreds or thousands of genes in any organism. In the following, I first give a brief account of Kacser and Burns's explanation of dominance in metabolic systems, and then discuss supporting experimental evidence for it.

### Robustness as a Systemic Property of Metabolic Systems

A mechanistic explanation of dominance in metabolic genes rests on one basic insight, namely that metabolic enzymes do not act in isolation, but are part of pathways or even larger networks of chemical reactions. The substrates of any one enzyme are the products of other enzymes. Conversely, the reaction products of any one enzyme are further transformed by yet other enzymes. Mutations in metabolic genes affect the activity of enzymes, and thus the rates at which a chemical reaction proceeds. In the mathematical description of metabolic systems, this rate—the amount of substrate converted into product per unit time—is usually called the *flux* through a reaction (150, p. 4). Flux is a key variable that links the activity of a metabolic enzyme to the phenotype. The reason is that the flux through some chemical reactions is strongly associated with fitness components like cellular growth or biomass production. This holds in particular for reactions that generate essential biomass components such as amino

acids, for reactions that produce high-energy phosphate bonds (ATP), or for reactions whose products have protective functions such as pigments.

Because some metabolic fluxes are highly correlated with fitness, dominance in metabolic genes is best expressed in terms of flux. To quantify the relation between enzyme activity and flux, one must ask the following question: How does the flux  $F$  through some critical chemical reaction change if the activity  $E_i$  of enzyme  $i$  somewhere else in the system changes (for example, by 50% or more)? The quantity that describes this change is known as the flux control coefficient  $C_i$ . To eliminate the dependency of  $C_i$  on the particular units in which enzyme activity and flux are measured, one can represent  $C_i$  as a fractional change, and thus as a dimensionless number:

$$C_i = \frac{\Delta F/F}{\Delta E_i/E_i} \quad (8.1)$$

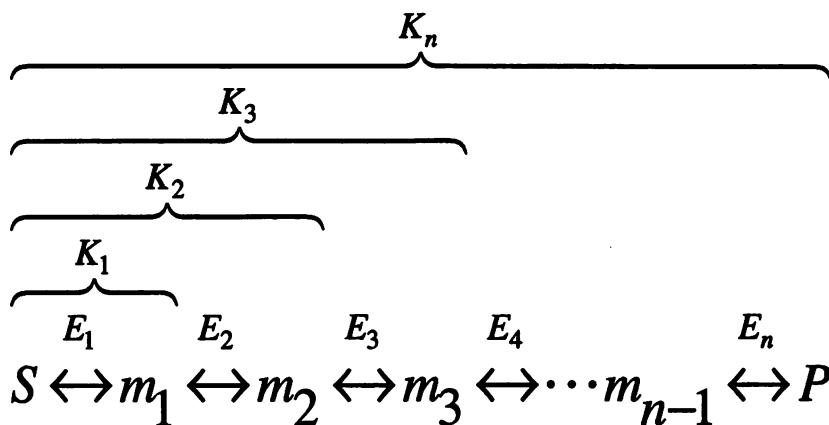
If a large change in enzyme activity  $E_i$  changes an important flux  $F$  by a small amount, then a metabolic system is robust to changes in enzyme concentration. For example, if flux is insensitive to a change in an enzyme's activity of 50% or more, the gene encoding the enzyme will show dominance to loss-of-function mutations in the enzyme. As defined in (8.1),  $C$  will depend on the magnitude of the change  $\Delta E_i$ . It is often more convenient to eliminate this dependence by defining the flux control coefficient as the sensitivity of flux to infinitesimal changes in enzyme activity. Thus, an alternative definition of flux is

$$C_i = \frac{\partial F/F}{\partial E_i/E_i} = \frac{\partial F}{\partial E_i} \frac{E_i}{F} \quad (8.2)$$

I note parenthetically that the notion of control coefficients is not restricted to linear metabolic pathways, yes, not even to metabolic control itself (481). For instance, analogous control coefficients can be defined for gene regulatory circuits (480).

As defined in (8.2), a flux control coefficient  $C_i$  close to one implies that a small change in enzyme activity causes an almost equal change in flux. In this sense, one can say that enzyme  $i$  controls the flux  $F$ , or that it is a “rate-limiting step” of a metabolic system. However, if  $C_i$  is close to zero, a small change in  $E_i$  changes flux by very little.

To what extent do individual enzymes control flux in a metabolic system comprising many reactions? Kacser and Burns (267) solved this problem for the simplest possible case of a linear metabolic pathway (Figure 8.1). The enzymes in the pathway are Michaelis-Menten enzymes, enzymes with especially simple kinetics and a hyperbolic relation between the



**Figure 8.1** A linear metabolic pathway comprising  $n$  Michaelis–Menten enzymes that catalyze  $n$  chemical reactions leading from some substrate  $S$  to some product  $P$  via intermediate metabolites  $m_i$ . The parameters  $K_i$  are the equilibrium constants of the summary reaction leading from  $S$  to metabolite  $m_i$ . They are properties of the reactions themselves, and thus independent of enzyme activities. After Scheme 1 of (267).

concentration of their substrates and their reaction rate (528). The pathway comprises  $n$  enzymes that catalyze  $n$  chemical reaction leading from some substrate  $S$  to some product  $P$  via intermediate metabolites  $m_i$ . The enzymes are assumed to be unsaturated by metabolites. That is, the enzymes operate far below their maximal velocity. The parameters  $K_i$  in Figure 8.1 are the equilibrium constants of the summary reaction between  $S$  and metabolite  $m_i$ . (Because enzymes influence only the approach of a reaction's equilibrium but not the equilibrium itself, the  $K_i$  are properties of the reactions and independent of the enzymes.) Under constant physiological conditions, metabolic systems must arrive at a steady state, otherwise individual metabolites would disappear, or their concentrations would increase beyond bounds. If metabolite concentrations vary cyclically, their time-averaged concentration have to approach a constant value, which can be viewed as the steady state. These observations justify analysis of the steady-state behavior of the pathway in Figure 8.1. In the steady state, the concentrations of individual metabolites  $m_i$  will not change and the overall flux from  $S$  to  $P$  will approach some constant value  $F$ . The value through each of the reactions must then also be equal to  $F$ , otherwise mass conservation would be violated. This flux can be calculated as (267)

$$F = \frac{M}{1/E_1 + 1/E_2 + 1/E_3 + \cdots + 1/E_n} \quad (8.3)$$

Here,  $M$  is a constant that depends on the concentration of  $S$  and  $P$ , as well as on  $K_n$ , but not on the activities of any enzyme in the pathway. The values  $E_i$  are proportional to enzyme activity. More precisely,  $E_i = (V_{\max, i}/K_{m, i})K_{i-1}$ , where  $V_{\max, i}$  is the maximal velocity of Michaelis-Menten enzyme  $i$ ,  $K_{m, i}$  is the Michaelis constant of enzyme  $i$  (the substrate concentration at which the half-maximal velocity  $(V_{\max, i})/2$  is attained), and  $K_{i-1}$  are the equilibrium constants of Figure 8.1 ( $K_0 = 1$ ).

Equation 8.3 clearly shows that if mutation eliminates any one enzyme ( $E_i \rightarrow 0$ ), the steady-state flux will be equal to zero. However, it also shows that steady-state flux depends on all enzyme activities. With expression 8.3 in hand, one can derive the flux control coefficient  $C_i$  (8.2) of each enzyme  $i$  by straightforward differentiation. This coefficient calculates as

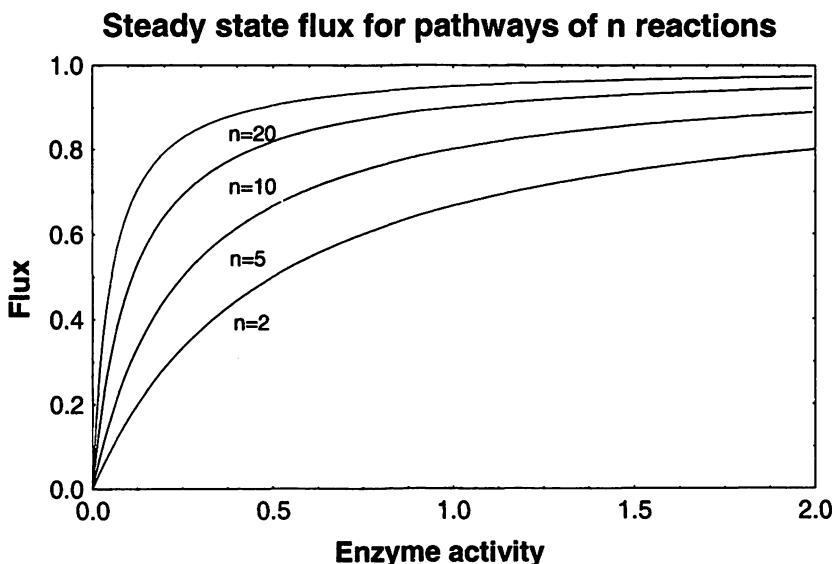
$$C_i = \frac{\partial F}{\partial E_i} \frac{E_i}{F} = \frac{1/E_i}{1/E_1 + 1/E_2 + 1/E_3 + \dots + 1/E_n} \quad (8.4)$$

and obeys the relation  $0 \leq C_i \leq 1$ . Equation 8.4 clearly demonstrates that flux control coefficients are systemic properties of chemical reaction pathways and cannot be understood by considering individual enzymes in isolation. Figure 8.2 shows the relationship of flux to the activity of any one enzyme in arbitrary units as a function of the number  $n$  of enzymes in the pathway. The flux control coefficient (8.2) is the tangent of the flux–activity curve at any one enzyme activity. Importantly, flux has a hyperbolic relationship to enzyme activity: As the activity of any one enzyme in the pathway increases, a small change in enzyme activity changes flux by an ever decreasing amount. This feature becomes ever more pronounced as the number of steps in a pathway increases. For example, if all enzymes in a pathway of  $n$  reactions have similar activities  $E_i$ , then the flux-control coefficient of any one enzyme equals  $C_i = 1/n$ . In long pathways, most enzyme activities may thus have negligible influence on pathway flux.

Equation 8.4 also reveals another property of flux-control coefficients in linear pathways. Their sum is one. This feature is known as the summation property (150):

$$\sum_{i=1}^n C_i = 1 \quad (8.5)$$

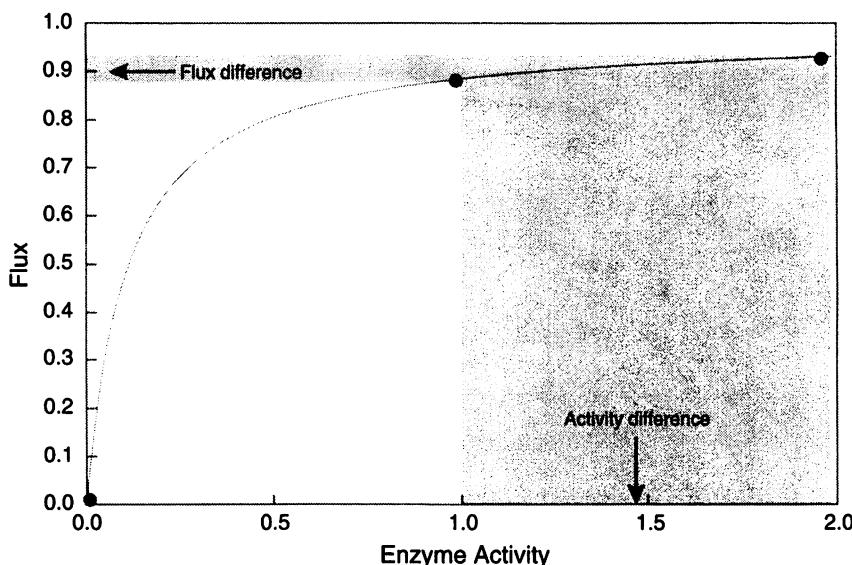
It implies that if one enzyme has a large flux-control coefficient, then the coefficients of all other enzymes must be close to zero. In this sense, flux control is a conserved quantity in a linear pathway.



**Figure 8.2** Steady-state metabolic flux in arbitrary units as a function of enzyme activity in arbitrary units, and of the number  $n$  of enzymatic reaction steps in a linear pathway. Fluxes were calculated with expression 8.3, where all enzyme activities but one were held constant at  $E_i = 1$ . One enzyme activity was varied from zero to two, as shown on the horizontal axis axis. To facilitate comparison, the maximally possible flux was set to one by choosing  $M = (n - 1)$  in (8.3).

Figure 8.3 illustrates how such distributed flux control can help explain the phenomenon of dominance in metabolic genes. As in Figure 8.2, the vertical axis shows flux as a function of enzyme activity (here for a pathway of 10 enzymes). The rightmost dot indicates the flux (phenotype) associated with a wild-type homozygote, an organism with two intact copies of an enzyme-coding gene. This homozygote has enzyme activity two (on an arbitrary scale). The leftmost dot indicates the flux of a null homozygote, which is equal to zero. The heterozygote (middle dot) has exactly intermediate enzyme activity. However, because of the hyperbolic relationship between activity and flux, the flux of the heterozygote is not intermediate between the two homozygotes. It is very close to the wild-type flux.

This systemic explanation of dominance rests on many simplifying assumptions, and it would need to be modified if these assumptions are not met (29, 30, 267, 481). The least problematic assumption is that enzymes are unsaturated, because many enzymes appear to be (150, chapter 4). Other assumptions include that enzymes have Michaelis–Menten kinetics (many enzymes show more complicated, nonlinear kinetics), that they act



**Figure 8.3** Flux reduction in a heterozygous null mutant. If the wild-type homozygote is on the plateau of the flux–activity curve, reducing activity by 50% in the heterozygous null mutant may reduce flux (and thus fitness) by a very small amount. Enzyme activities and fluxes are given in arbitrary units as in the previous figure. The flux–activity relation is shown for a pathway of  $n = 10$  enzymes.

in one linear pathway (many pathways are branched or cyclic), and that any control mechanism such as feedback inhibition is absent. Although many insights of linear pathway analysis carry over to more complicated scenarios, such as branched pathways, moderate nonlinearities in enzyme activities, and non-steady-state behavior (251, 275), when the simple assumptions no longer hold, deviations from these simple predictions may occur. For example, in branched pathways individual enzymes may show negative control coefficients, because increasing enzyme activity in one branch may decrease flux through the other branch. One may even see switch-like behavior, where small changes in an enzyme activity lead to a complete reallocation of flux between branches (319). It is difficult to model flux control coefficients in many of these more complex situations. However, much empirical evidence—some of it summarized below—suggests that the main predictions of the simplest case apply to a wide variety of situations.

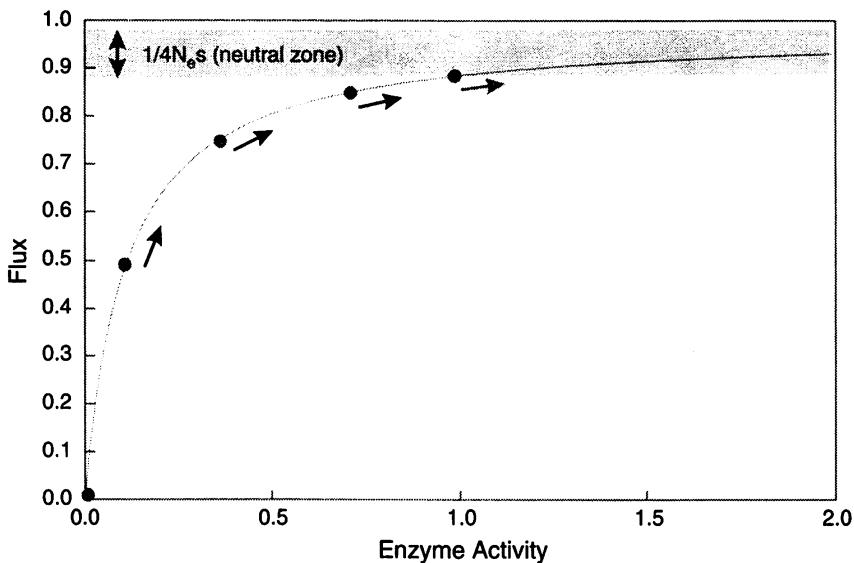
A final note on the role of regulatory mechanisms is in order. Most regulatory mechanisms would, if anything, further contribute to robustness and dominance. The reason is that most such mechanisms ensure homeostatic pathway output when substrate concentrations change. Take the

example of feedback inhibition, where the end product  $P$  of a pathway inhibits an intermediate enzyme  $i$ . Reducing—through a mutation—the activity of another enzyme in the pathway will reduce the rate at which the end product  $P$  of the pathway accumulates. The resulting reduced concentration of  $P$  will reduce the inhibition of enzyme  $i$ , which may then compensate in part for the reduced activity due to the mutation. Others have discussed the complexities of control in biochemical pathways in great detail (150, chapter 7, 480). However, this simple example encapsulates a very general point: under most circumstances, control mechanisms that ensure homeostasis to physiological and environmental changes will also enhance genetic robustness.

## Evolution of Robustness in Linear Pathways

Thus far, I have not even considered how evolutionary pressure on pathway flux may affect robustness. Such pressure may further contribute to robustness of flux against changes in enzyme activity, as Hartl and collaborators pointed out (223). Consider an enzyme that is part of a linear pathway whose steady-state flux is proportional to fitness (Figure 8.4). Natural selection will thus act to increase flux through the pathway. If the enzyme's activity is on the steep (left) part of the activity–flux parabola, then natural selection will favor alleles with increased activity, and thus push the enzyme's activity toward the plateau on the right of the curve. As a result, enzyme activity will approach ever-flatter parts of the plateau. This means that the enzyme's flux control coefficient becomes ever smaller. Put differently, the further out the enzyme is on the flux plateau, the lesser a given change in enzyme activity affects flux. This basic insight has an interesting evolutionary consequence that emerges from Kimura's neutral theory of molecular evolution (222, 285).

One of the key predictions of the neutral theory of molecular evolution relates the fitness effects  $s$  of a mutant—here a change in flux due to a changed enzyme activity—to the size  $N$  of a population. For a population of  $N$  sexually reproducing diploid organisms, mutations that cause a fitness change of much less than  $s = 1/4N$  are effectively neutral. That is, their frequency evolves under the influence of random genetic drift—driven only by random effects of sampling individuals from generation to generation—and not under that of selection. (Loosely speaking, natural selection does not “see” such mutants.) To be precise, one must replace the actual number of individuals  $N$  in this relation with the effective population size  $N_e$ . The effective population size reflects various life history features of actual populations. For example, usually not all individuals produce offspring, thus reducing the size of the gene pool that effectively



**Figure 8.4** Natural selection for increased flux increases robustness. Selection, acting on variation in one enzyme, increases enzyme activity. As a by-product, flux becomes less sensitive to changes in activity. Eventually, a plateau in the flux–activity curve will be reached where the average mutational change in enzyme activity affects flux by so little that the mutation is effectively neutral. At this point, selection for increased flux is no longer effective, and most mutations that decrease flux will also be neutral. After Figure 1 of (223).

contributes to the next generation. The calculation of  $N_e$  from  $N$  is discussed for specific scenarios in standard texts (222).

How much smaller than  $1/4N_e$  must a selection coefficient  $s$ —the fitness difference of mutant to wild-type—be such that the mutant is neutral? As a rule of thumb

$$\begin{aligned} \text{Genetic drift dominates if } & 4N_e s < \frac{1}{10} \quad \text{or} \quad s < \frac{1}{10(4N_e)} \\ \text{Natural selection dominates if } & 4N_e s > 10 \quad \text{or} \quad s > \frac{10}{4N_e} \end{aligned} \quad (8.6)$$

In between these two regimes, both genetic drift and natural selection influence the frequency of a mutant allele.

What is the consequence of an enzyme's ever-decreasing effect on flux as its activity increases in evolution? Enzyme activity will approach what Hartl and collaborators termed a neutral zone (223). This is an area of the flux–activity curve where the average mutational change in enzyme

activity—say,  $x$  percent—is effectively neutral with respect to flux and fitness. The location of this neutral zone will depend both on the extent  $x$  to which mutations change enzyme activity on average and on the population size. The larger the population, the higher enzyme activity must have become before the neutral zone is reached. However, once the neutral zone is reached, selection will not be effective in increasing flux further. The reason is that most mutations that increase enzyme activity, unless they increase activity manifold, will be neutral, much like most mutations that decrease enzyme activity. This effect is enhanced by the flattening of the activity–flux curve with increased activity. (The flattening means that an increase in enzyme activity by  $x$  percent increases flux by a lesser amount than an activity decrease by  $x$  percent decreases flux.)

In sum, robustness of metabolic pathways to changes in enzyme activity, although a consequence of distributed flux control, can be further enhanced if natural selection acts to increase flux through an increase in enzyme activity. Provided that sufficient genetic variation in enzyme activity is available, this increase will continue until a neutral zone is reached. Even drastic reductions in enzyme activity, such as seen in heterozygous loss-of-function mutations, will then have slight effects on flux. In other words, flux has become even more robust to changes in enzyme activities.

### Evidence: Flux Measurements

For many organisms—including humans and their metabolic diseases—wild-type alleles of metabolic genes are dominant. Unfortunately, the relationship between metabolic flux and enzyme activity has rarely been measured. Notable exceptions include the flux through ethanol catabolism as a function of alcohol dehydrogenase activity in *Drosophila*, and the measurement of arginine synthesis flux in the fungus *Neurospora crassa* as a function of ornithine carbamoyltransferase activity and arginino succinate activity (159, 160, 373). In these cases, the flux–activity relation shows the expected hyperbolic relation (Figure 8.3), and a 50% reduction in enzyme activity changes flux by very little.

### Evidence: Robustness as a Systemic Property

Is the systemic nature of distributed flux control—emerging from laws of chemical kinetics—mainly responsible for robustness in metabolic genes, or does natural selection contribute substantially to this robustness? It is usually impossible to tease these two factors apart. However, in one organism, the unicellular algae *Chlamydomonas reinhardtii*, some evidence

speaks to this question. *Chlamydomonas reinhardtii* spends the overwhelming part of its life cycle in the haploid state. It can proliferate vegetatively in this state. However, it can also reproduce sexually. After gamete fusion, its zygotes usually undergo meiosis, but in rare cases they divide vegetatively in the diploid state. In such diploid cells, one can then assess whether wild-type alleles are dominant over mutant alleles. Such dominance could not be explained through selection, because *Chlamydomonas* spends most of its life cycle in the haploid state. Orr (419) surveyed evidence on dominant genes in *Chlamydomonas*. He found that more than 90% of genes for which the necessary information was available had recessive mutants, a value very similar to that found in a diploid organism like *Drosophila melanogaster*. To be sure, many of these dominant wild-type genes do not encode metabolic enzymes. However, if dominance in metabolic genes is as pervasive as in the surveyed genes, then one would conclude that dominance in metabolic genes does not require natural selection either. Systemic properties may thus account for many dominant wild-type alleles in this organism.

### Evidence: Frequency of Null Alleles

In a null allele, the product of a gene has lost its function. The frequency  $q$  at which a null allele occurs in a population can be used to estimate by how much fitness is reduced in a heterozygote involving this null allele (222). The reason is that the following relation holds between heterozygote fitness and null-allele frequency. If the homozygous null genotype has a fitness  $(1 - s)$  relative to the wild-type, if the heterozygote has a fitness of  $(1 - hs)$ , and if a wild-type allele mutates to a null allele at a rate  $\mu$ , then the equilibrium frequency of the allele in mutation-selection balance is approximated by (222, p. 237)

$$q = \frac{\mu}{hs} \quad (8.7)$$

$q$  and  $\mu$  can sometimes be estimated, such that one can calculate  $hs$ , the reduction of fitness in heterozygotes relative to the wild-type. A case in point are null alleles at the alcohol dehydrogenase (*Adh*) gene in *Drosophila melanogaster*. Using empirical estimates of mutation rates and equilibrium allele frequencies for this gene, Hartl and collaborators (223) determined that  $hs = 0.0037$ . That is, the fitness of heterozygotes for the null allele is only slightly less than that of the wild-type, despite the fact that enzyme activity is cut in half. From these values, they could also estimate the average increase in fitness  $s$  caused by a 1% increase in *Adh* activity. It is very small ( $s = 1.8 \times 10^{-5}$ ). Given population size estimates of  $N_e = 10^4$

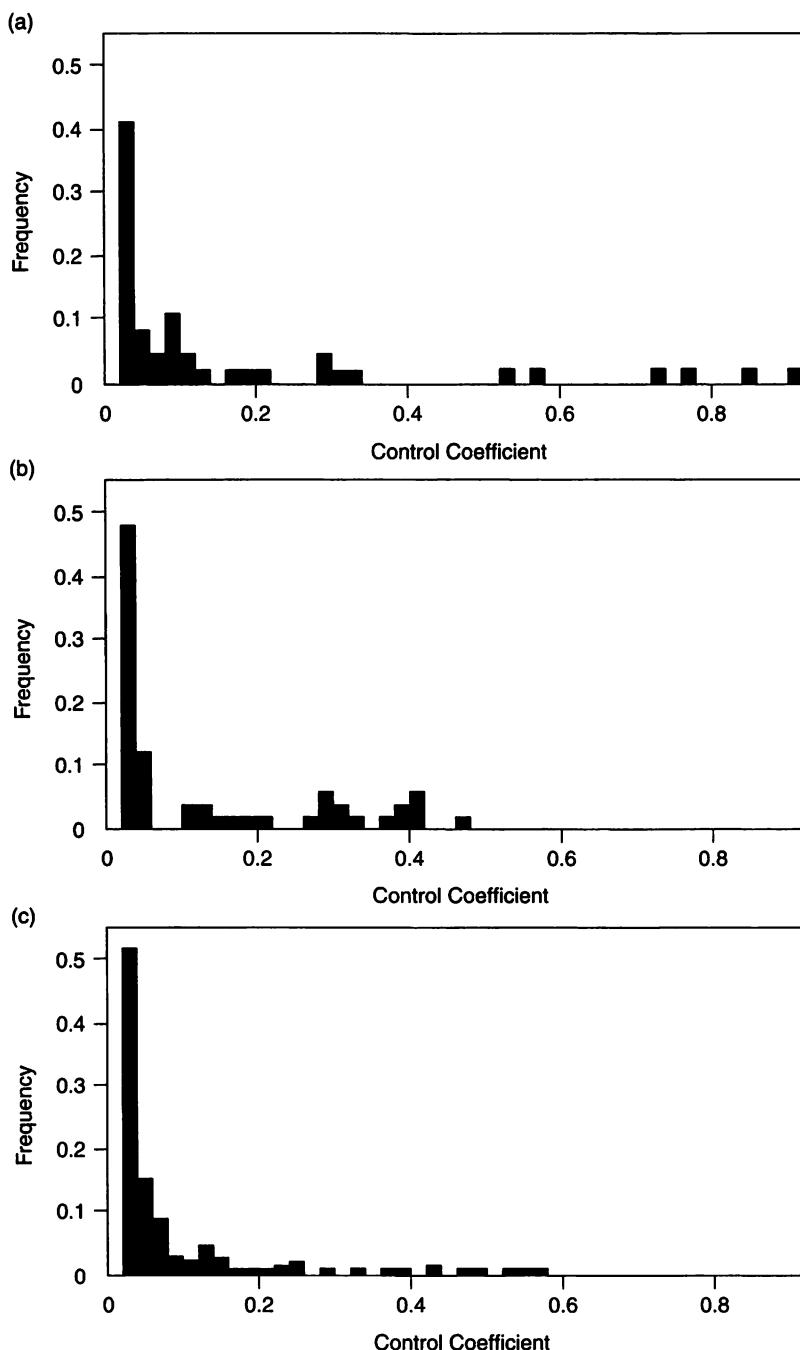
for *Drosophila* populations in North America, such an increase is probably neutral. Hartl and collaborators derive similar estimates for 14 other *Drosophila* loci (223). They find invariably that fitness reduction in heterozygotes is small (an average of  $hs = 0.0015$ ). Increases in enzyme activity by 1% would increase fitness by an average of  $s = 7.4 \times 10^{-6}$ , and would thus be neutral in a population of the above size.

### Evidence: Enzyme Activities in *Escherichia coli*

Microbes are ideal organisms to study the effects of mutations on fitness of growing cells, because one can study mutational effects in them through competition assays. The basic concept is simple. Mix mutant and wild-type cells in equal proportions and let the cells divide in a defined medium for a specified amount of time or generations. The final proportion of mutant to wild-type cells provides an estimate of the fitness difference between mutants and wild-type (106). In a prokaryotic microbe like *E. coli*, the notion of dominance is problematic, because the organism is not diploid. However, one can still determine the effects of changing enzyme activities on fitness. Dykhuizen and collaborators (127) did so for two enzymes necessary to metabolize the sugar lactose, lactose permease and  $\beta$ -galactosidase. Lactose permease is necessary for lactose uptake into the cell, whereas  $\beta$ -galactosidase metabolizes lactose into sugars that central metabolism can readily use. If lactose is the sole carbon source available to the cell, flux through these two reactions is proportional to growth rate and thus fitness. Experiments confirm the prediction that the relationship between either enzyme and fitness is hyperbolic (Figure 8.4). The control coefficient of  $\beta$ -galactosidase is exceedingly small ( $C = 0.018$ ), because enzyme activity in wild-type cells is on the plateau of the flux activity curve (127). As a result, even a 75% reduction of  $\beta$ -galactosidase activity results only in a 1.3% reduction in fitness (127). On the other hand, permease activity has a larger control coefficient of  $C = 0.551$  and its activity is on the shoulder of the flux-activity curve. The reason for the low activity of this transport protein may lie in the undesirable side effects of increasing its activity. For instance, an activity increase may render a cell vulnerable to osmotic shocks if sugar concentrations change drastically (105).

### Evidence: Distribution of Multiple Flux Control Coefficients

The most elementary predictions of metabolic control analysis are simple. For example, the summation property shows that the average flux control coefficient in a pathway of  $n$  equally active enzymes equals  $(1/n)$ .



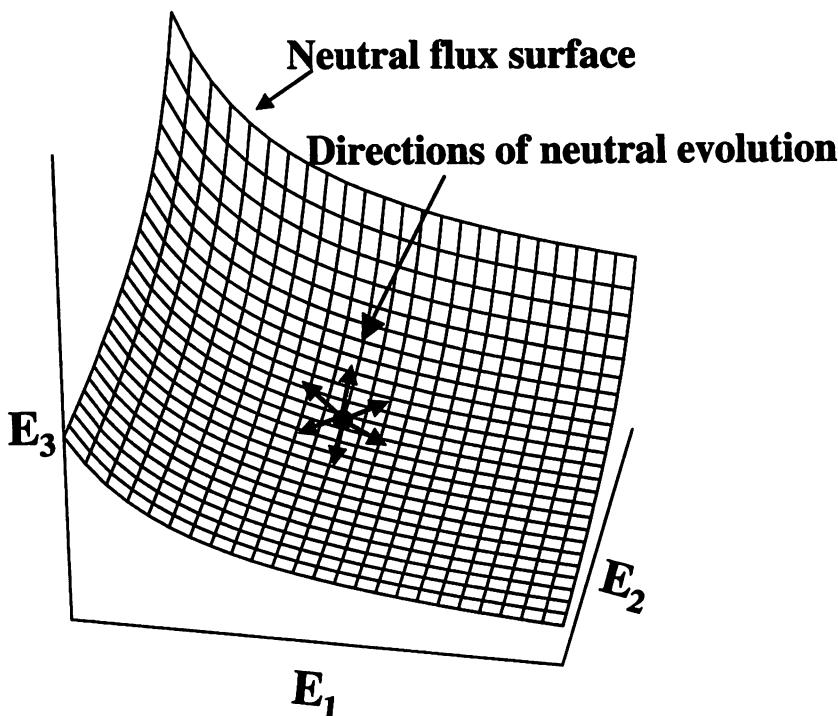
However, the theory does not make a prediction about the distribution of enzyme activities and flux control coefficients in actual pathways. It may well be that a few coefficients are much larger than  $(1/n)$ , whereas others are much smaller, as the previous example indicates. It is also not clear how evolution would shape the distribution of flux control coefficients. The concept of evolution toward neutrality is based on changes in the activity of one enzyme. What happens if evolution changes the activity of many enzymes simultaneously? Certainly, not all enzymes can evolve ever-smaller flux control, because the sum of the control coefficients must be one. But would this distribution be uniform or not? This question has been addressed by de Vienne and collaborators (56) who predict—based on a quantitative genetic model of flux control—that flux control coefficients should show a skewed distribution, where most enzymes in a pathway have very small flux control coefficients, and only a few have large flux control. Empirical evidence supports their predictions. Figure 8.5 shows a compilation of such evidence for three different metabolic systems, including rat gluconeogenesis and succinate oxidation in cucumber mitochondria (8, 213, 232). The majority of enzymes show small flux control coefficients of  $C < 0.05$ . Flux will be very robust to changes in the activity of these enzymes.

### Flux Control in Evolutionary Time: The Role of Neutral Spaces

These snapshots of the distribution of robustness at different loci leave one important question open. How do flux control coefficients change in evolutionary time? Would the observed skewed distribution of flux control coefficients remain static, such that the flux control of each individual enzyme remains unchanging? Or would the activities of individual enzymes keep changing, such that only the overall shape of the distribution remains constant? The answer will depend in part on how mutations affect enzyme activities, but I speculate that the latter will be the case. That is, because the summation property (8.5) constrains only the sum of the control coefficients, it leaves enormous space—a “neutral” space—for continuing

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**Figure 8.5** Flux-control coefficients follow a distribution skewed toward small flux control coefficients. The figure shows empirical data compiled by de Vienne and collaborators (56). (a) Flux-control coefficients of 8 steps of gluconeogenesis in isolated rat liver cells under various experimental conditions. Data from (213). (b) Flux-control coefficients for 5 steps of succinate oxidation in isolated cucumber mitochondria. Data from (232). (c)  $\text{CO}_2$  flux-control coefficients for 26 steps of a computer model of the tricarboxylic acid cycle in *Dictyostelium discoideum*. Data from (8).



**Figure 8.6** A surface of constant flux in a pathway of three enzymes. Changing enzyme activities along the surface does not change flux and is thus neutral. The surface was calculated from equation 8.3 under the constraint of constant flux.  $M/F = 10$ , and enzyme activities  $E_1$  and  $E_2$  range from 0.5 to 2 in arbitrary units.

change in enzyme activities. Enzyme activities can drift freely in this space, as long as the overall flux through a pathway does not change. Enzymes that have very small flux control at one point in time may well attain large flux control many generations later, and vice versa. Put differently, at one point in time flux may be nearly immune to activity changes in a particular enzyme, whereas many generations later, it may become exquisitely sensitive. The caricature of Figure 8.6 illustrates a surface of neutral flux derived from (8.3) for a three-enzyme pathway. Along this surface, changes in enzyme activity do not change flux. It is best to think of this surface not as being infinitely thin, but as a sheet in three-dimensional space of thickness  $1/20N_e$ , simply because deviations in flux smaller than  $1/40N_e$  above and below this surface would be effectively neutral. For longer pathways, this two-dimensional surface becomes an  $(n - 1)$ -dimensional neutral space of flux change. In this space, enzyme activities can change neutrally, such that individual flux control coefficients may continually change in time.

An observation relevant to this scenario is that many enzyme-coding genes show polymorphisms in natural populations that affect their activity. Selection has acted on these polymorphisms at some point in the past (128). (Unfortunately, it is, with few exceptions, unknown how strongly naturally occurring enzyme variants affect flux.)

Two main factors can cause changing selection pressures on enzyme polymorphisms. The first is environmental change and the second is drift of flux control through a neutral space. With environmental change, the demands on flux through any one metabolic pathway can change. This also means that the flux control of individual enzymes—as well as the robustness of flux—may change. An experimental example is the gene for 6-phosphogluconate dehydrogenase, an enzyme necessary to metabolize the carbon source gluconate. A mutant *E. coli* cell in which the amount of the protein is increased by 300% relative to wild-type shows a drastic (57%) increase in fitness if gluconate is the sole carbon source (223). However, if glucose is the sole carbon source, the organism's growth rate is not increased. Another example comes from the photosynthetic enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase-oxygenase) in tobacco. Rubisco's flux control coefficient can vary by more than an order of magnitude among plants grown in different lighting conditions (320).

Importantly, the second factor that may cause varying selection pressures on enzyme polymorphisms, the—still hypothetical—evolutionary drift of flux control, occurs even in an unchanging environment. If such drift occurs, a once neutral enzyme variant (with changed activity) may come under the influence of selection and vice versa. Consistent with this prediction is accumulating evidence on the episodic and ephemeral nature of selection on enzyme variation. A particular enzyme variant may attain a high population frequency, only to disappear shortly thereafter, and polymorphisms in enzyme activity that persist for long times are generally rare (37, 128, 129, 225, 246). Whether environmental change or evolutionary change in flux control contribute predominantly to such patterns of molecular evolution is an important and open question.

In sum, robustness of metabolic pathway flux and fitness to changes in enzyme activities are pervasive. Both systemic properties of metabolic pathways and natural selection for increased enzyme activity are responsible for such robustness. In metabolic pathways of many enzymes, a large neutral space exists in which enzyme activities can drift. This drift and/or changing environments cause selective pressure to shift among enzymes of a metabolic system.

# 9

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## Metabolic Networks

What is robust? The flux of matter through a metabolic network and cell growth.

What is it robust to? Drastic decreases and increases in flux through individual chemical reactions, and complete elimination of such reactions.

The metabolic pathways of the previous chapters are but figments of the complex chemical reaction networks sustaining life. To understand robustness of metabolism ultimately requires understanding such larger metabolic networks. Metabolic control analysis, whose basic principles were discussed in the previous chapter, can be used to study such networks, but it also faces severe practical limitations (150). First, applying metabolic control analysis to large reaction networks comprising thousands of reactions poses formidable mathematical problems. Second, metabolic control analysis requires much quantitative information, in particular about the rate at which enzymatic reactions proceed inside cells. Unfortunately, with the exception of a few simple model systems, such as the metabolism of human red blood cells (428), such information is unavailable. This holds even for the best-studied metabolic networks, such as that of *Escherichia coli*: Although most chemical reactions catalyzed by enzymes in this bacterium are known, kinetic information is available for only a small fraction of these enzymes. These problems raise the question: Is it possible to characterize large chemical reaction networks and their robustness even though information about the reaction rates of many enzymes is unavailable? The answer is yes. It can be done by merely examining the stoichiometric properties of metabolic networks, using related approaches such as flux balance analysis (134) or elementary mode analysis (492, 494). I will briefly outline the foundation of these approaches and some of the insights they provide. These insights are complementary to those of the previous chapter. They indicate that metabolic networks are very robust to mutations and they teach us an important lesson: Our representation of a biological system critically influences what we can learn about its robustness.

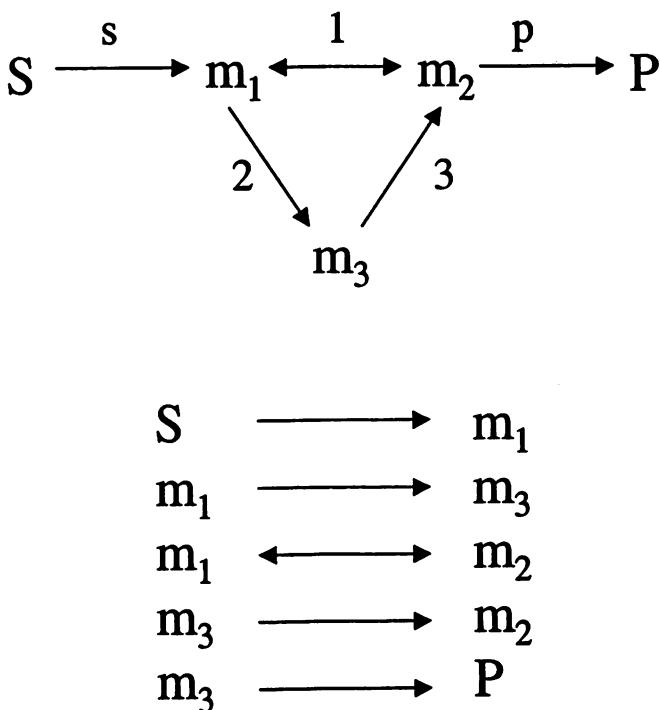


Figure 9.1 A simple chemical reaction scheme where an external substrate  $S$  is transformed into a metabolic product  $P$  via a series of chemical reactions involving internal metabolites  $m_i$ . The scheme is represented in two equivalent forms.

### Background on Stoichiometric Network Analysis

Consider the following simple reaction scheme (Figure 9.1), where some substrate  $S$  external to the cell is imported and/or converted into some metabolite  $m_1$ , which can then be converted through a reversible reaction (indicated by a double-headed arrow) into metabolite  $m_2$ .  $m_2$ , in turn, is a precursor to some product  $P$ .  $P$  might be a biomass component essential for cellular growth, a product the cell secretes into its environment, or a waste product. Alternatively to its direct interconversion with  $m_2$ ,  $m_1$  can also be converted into  $m_2$  through a chain of two irreversible chemical reactions involving a metabolite  $m_3$ .

As in the previous chapter, the most important variables in a stoichiometric analysis of chemical reaction networks are the amounts of matter that flow through each of the chemical reactions indicated by the arrows

in Figure 9.1. These are the network's metabolic fluxes  $v_i$ , where the subscript  $i$  corresponds to the name or index of the chemical reaction, as written above the arrows in Figure 9.1. The changes in concentrations of the metabolites  $m_i$ ,  $dm_i/dt$ , are simple functions of these fluxes. For the example of Figure 9.1, they can be written in terms of the following simple system of differential equations:

$$\begin{aligned}\frac{dm_1}{dt} &= v_s - v_1 - v_2 \\ \frac{dm_2}{dt} &= v_1 + v_3 - v_p \\ \frac{dm_3}{dt} &= v_2 - v_3\end{aligned}\tag{9.1}$$

Metabolic "inputs" like  $S$  and "outputs" like  $P$  are usually referred to as "external" metabolites. The changes in their concentrations are not explicitly modeled and are distinguished from "internal" metabolites  $m_i$ , whose interconversion constitutes metabolism proper. The internal metabolites are subject to conservation of mass, which is reflected in the fact that by summing all derivatives  $dm_i/dt$  the internal fluxes  $v_i$  cancel. That is, one is left only with fluxes from and to external metabolites:

$$\frac{dm_1}{dt} + \frac{dm_2}{dt} + \frac{dm_3}{dt} = v_s - v_p$$

Under constant environmental conditions, the changes in metabolite concentrations  $dm_i/dt$  must approach zero, otherwise some metabolites would disappear completely, whereas the concentration of others would approach infinity. In this steady state, individual metabolite concentrations do not change. The steady state is dynamic, because in it constant metabolite concentrations are maintained by ongoing interconversions of metabolites. Transient changes in metabolite concentrations can of course occur, for example when an environment changes, but metabolic networks reach their new steady state after such changes often very rapidly, typically within minutes (429). In addition, even if metabolite concentrations sometimes show more complex behavior, such as sustained oscillations, the time-averaged metabolite concentrations are constant, and thus the network is effectively in steady state. For these reasons, it is thus often sensible to focus on the steady-state properties of equation 9.1, which simplifies the mathematical treatment of a metabolic network considerably. This is illustrated by the following simple example, which

shows how to calculate the metabolic fluxes allowed in the steady state. I begin by writing (9.1) in a more compact form, i.e.,

$$\frac{d\bar{m}}{dt} = S\bar{v} \quad (9.2)$$

This linear differential equation is just a different way of representing equation 9.1.  $\bar{m} = (m_1, m_2, m_3)$  is the vector of internal metabolites.  $\bar{v} = (v_s, v_1, v_2, v_3, v_p)$  is the vector of all fluxes, and  $S$  is a matrix that contains the coefficients of this differential equation, which are simply the stoichiometric coefficients of the chemical reactions for each metabolite  $m_i$ . For the simple reaction scheme of Figure 9.1, this matrix has the following structure:

$$S = \begin{pmatrix} 1 & -1 & -1 & 0 & 0 \\ 0 & 1 & 0 & 1 & -1 \\ 0 & 0 & 1 & -1 & 0 \end{pmatrix} \quad (9.3)$$

Its columns correspond to the reactions  $s$ , 1, 2, 3, and  $p$  of Figure 9.1. Its rows correspond to metabolites  $m_1$ ,  $m_2$ , and  $m_3$ . Positive and negative signs of matrix entries indicate whether a metabolite occurs in the left or right side of a chemical reaction in Figure 9.1b. For example,  $S_{32} = 1$ , because metabolite  $m_3$  is a product (occurs on the right-hand side) of reaction 2. In the notation of (9.2), the steady-state condition, where no changes in internal metabolite concentrations occur, can be written as

$$\frac{d\bar{m}}{dt} = 0$$

which is equivalent to

$$S\bar{v} = 0 \quad (9.4)$$

For the simple example of Figure 9.1, this condition is equivalent to

$$\begin{aligned} v_s - v_1 - v_2 &= 0 \\ v_1 + v_3 - v_p &= 0 \\ v_2 - v_3 &= 0 \end{aligned} \quad (9.5)$$

As a mere matter of convention, the fluxes corresponding to external metabolites are sometimes written on the right-hand side of (9.5), such that one obtains

$$\begin{aligned} -v_1 - v_2 &= -v_s \\ v_1 + v_3 &= v_p \\ v_2 - v_3 &= 0 \end{aligned} \tag{9.6}$$

This can be written again in matrix form

$$S' \bar{v}' = \bar{b} \tag{9.7}$$

where the stoichiometry matrix  $S'$  is

$$S' = \begin{pmatrix} -1 & 0 & 1 \\ 1 & 0 & 0 \\ 0 & 1 & -1 \end{pmatrix}$$

and the vector  $\bar{v} = (v_1, v_2, v_3)$ , and  $\bar{b} = (-v_s, v_p, 0)$ . The equivalence of (9.5) and (9.6) shows that the two matrix formulations also have equivalent solutions. However, there is a conceptual difference. In (9.4), all fluxes, including those from and to the external metabolites  $v_s$  and  $v_p$ , are treated as variables and the steady-state flux distribution is obtained by solving for  $\bar{v}$ . In (9.7), these external fluxes are absorbed into the vector  $\bar{b}$  and are thus treated as constants. One can think of them as availabilities of a substrate in the environment, or as constant export rates of a product. One thus solves (9.7) only for the three internal fluxes as variables. The solution is a vector  $\bar{v}$  of allowed internal steady-state fluxes, fluxes that do not violate the law of mass conservation. In sum, all this means that the steady states of metabolic networks are described by simple systems of linear equations such as (9.7). To solve these equations is to identify the fluxes allowed in steady state.

I note that the above stoichiometry matrices  $S$  and  $S'$  are unusual in several respects. First, and trivially, they contain many fewer reactions than are encountered in any network of realistic complexity. Second, the reaction scheme of Figure 9.1 contains only monomolecular reactions. Each of these reactions has only one educt and only one product. Inside living cells, bimolecular reactions, where two chemical compounds react, are much more frequent. Such reactions, however, are easily incorporated into a stoichiometry matrix  $S$ . Each column of  $S$  (corresponding to one reaction) can carry as many negative entries as the reaction has educts, and as many positive entries as it has products. Third,  $S$  in this example

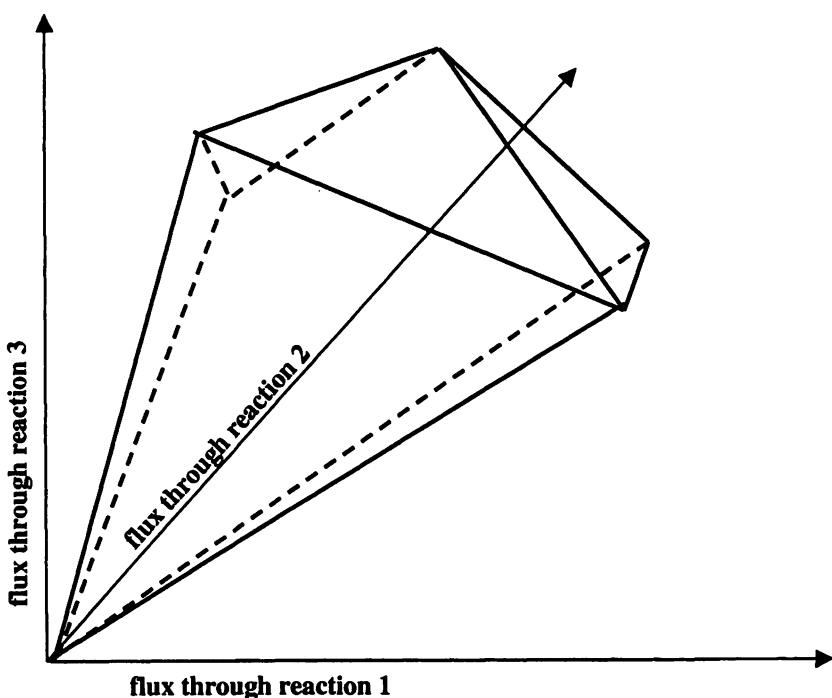
contains only nonzero entries (+1) and (-1), but many reactions do not convert molecules in equimolar proportions. This complication is also easily incorporated. For example, if reaction 3 needed two molecules of  $m_3$  to produce one molecule of  $m_2$ , then  $S'_{33}$  would be equal to (-2) instead of (-1).

In addition to the flux-balance condition (9.4) in the steady state, any metabolic reaction network has to fulfill several additional constraints. First, fluxes cannot become arbitrarily small or large, such that they need to be bounded between some real values. The reason is that only a limited amount of any one enzyme can be present, and that enzymes catalyze reactions at finite rates. Second, some reactions are irreversible and can proceed in only one direction. By convention, the respective flux  $v_i$  cannot be negative. The same principles hold for the import of an external metabolite, such as a carbon source, or for the export of a metabolic end product. The respective flux has a maximal rate, which reflects factors such as the concentration of a metabolite in the environment and the transport mechanism of the metabolite.

## Main Tasks of Stoichiometric Network Analysis

Stoichiometric analysis has two main tasks. First, it identifies the allowed flux vectors  $\bar{v}$  that fulfill all the constraints on the network. These include the steady-state condition (9.4), as well as the additional constraints on flux magnitudes and signs I just listed. Flux vectors that meet these conditions are “allowed” fluxes, fluxes that a cell can realize without violating the law of mass conservation. There is almost never just one unique allowed flux vector. The reason is that in most metabolic reaction networks with  $n$  internal metabolites and  $m$  chemical reactions, there are many more chemical reactions than metabolites ( $m \gg n$ )—many fewer equations than variables—such that (9.4) is massively underdetermined. If metabolism was under no constraints other than (9.4), the set of allowable fluxes would be an  $(n - m)$ -dimensional (vector) space, which is also called the null space of the stoichiometry matrix  $S$ . However, because fluxes cannot exceed a maximally possible value, the allowable flux vectors occupy a bounded region in this space—a high-dimensional cone of allowed fluxes—as indicated in the three-dimensional caricature of Figure 9.2.

The second task of stoichiometric analysis is to identify regions within the set of allowable fluxes that maximize a desirable property. One example of such a property is cell growth, which is an indicator of fitness under conditions where cells grow exponentially. Consider a genetically heterogeneous population of single-celled organisms that actively grow



**Figure 9.2** A schematic representation of allowed steady-state fluxes for a hypothetical set of three chemical reactions (not shown). The figure mainly illustrates that the allowed fluxes do not form a vector space but instead form a bounded subset (flux cone) of a vector space, the null-space of a stoichiometric matrix  $S$ .

and divide. Each cell or genotype in this population may occupy a different position in the region of allowed metabolic fluxes, because its enzymes and their expression levels under any particular environmental condition differ from those of other cells. Those cells in the population that grow at a maximal rate will outgrow all other cells and thus come to dominate the population. For such maximal cell growth, biosynthetic precursors such as amino acids need to be made in well-defined proportions. Similarly, high-energy phosphate bonds (ATP and related molecules) and redox potential (NADH and related molecules) need to be produced in balanced amounts. This means that the metabolic fluxes generating them must have particular values, which can be identified if the optimal proportions of biosynthetic precursors, energy carriers, and redox potential are known. In some well-studied organisms, such as the bacterium *Escherichia coli*, these proportions are known from the biomass

composition of the organism (169). In trying to find the flux vectors  $\vec{v}$  that yield maximal growth under any one environmental condition, one tries to identify one or more points in the realizable region of the space of fluxes (Figure 9.2) that maximize or minimize some function  $Z$  of the flux  $\vec{v}$ . In practice, linear functions of  $\vec{v}$  are most important, such that

$$Z(\vec{v}) = \sum_{i=1}^m c_i v_i \quad (9.8)$$

and one tries to find values of  $\vec{v}$  that maximize  $Z$ . In practice, the function  $Z$  is maximized by standard numerical techniques such as linear programming. As an aside, finding fluxes that ensure maximal growth is by far not the only application of this approach. Others include identification of sustainable fluxes that minimize ATP production—corresponding to energy-efficient growth—or identification of fluxes that produce maximal amounts of an industrially important metabolite, such as an antibiotic. The approach is thus of great relevance for metabolic engineering of organisms in industrial biotechnology. Importantly for my purpose, there are usually many different flux vectors  $\vec{v}$  that maximize the same property (represented by  $Z$ ). In other words, there is a large neutral space of fluxes associated with many biological properties of interest.

Note that even though particular points in realizable flux space may be identified as optimal for cell growth, it is by no means assured that a cell can achieve the desired metabolic fluxes. For example, when faced with certain carbon sources, a cell may not be able to express the required enzymes in the amounts that assure optimal growth. A case in point is the *E. coli* strain MG1655, which grows on glycerol as sole carbon source at a rate lower than predicted as optimal by this approach. However, within 40 days or 700 generations of growth on glycerol in the laboratory, the strain increases growth substantially and the fluxes approach those predicted by theory as optimal (249). This shows not only the power of this approach to predict optimal flux distributions, but also the power of natural selection to achieve the appropriate patterns of gene regulation within short amounts of time.

To summarize so far, stoichiometric analysis of large enzymatic reaction networks can identify realizable metabolic fluxes, fluxes that fulfill certain boundary conditions. Within this set of all realizable fluxes, it can identify fluxes that endow a cell with properties of interest. An important property for evolutionary studies is that of maximal cell growth, because it is an indicator of fitness under conditions where cells actively divide.

## Applications to Robustness

The approach outlined above is suitable to analyze robustness of cell growth to changes in individual fluxes. That is, under conditions where cells grow maximally, one can ask: What are the effects of changing only one individual flux drastically, such as by forcing it to assume a value of zero? Biologically, such a change would correspond to a loss-of-function mutation in an individual enzyme catalyzing a particular reaction, or in a loss of the enzyme's expression. Thus, the robust feature here is a particular distribution of metabolic fluxes and the corresponding growth rate of a single-celled organism, an indicator of fitness. What is it robust to? Reduction of flux through individual reactions, that is, loss-of-function in individual enzyme-coding genes. Because the approach is computational, it is easily possible to determine robustness to changing—one by one—all fluxes in a network. One can do the same even for all flux pairs, triplets, etc.

## Robustness to Complete Elimination of Enzymatic Reactions

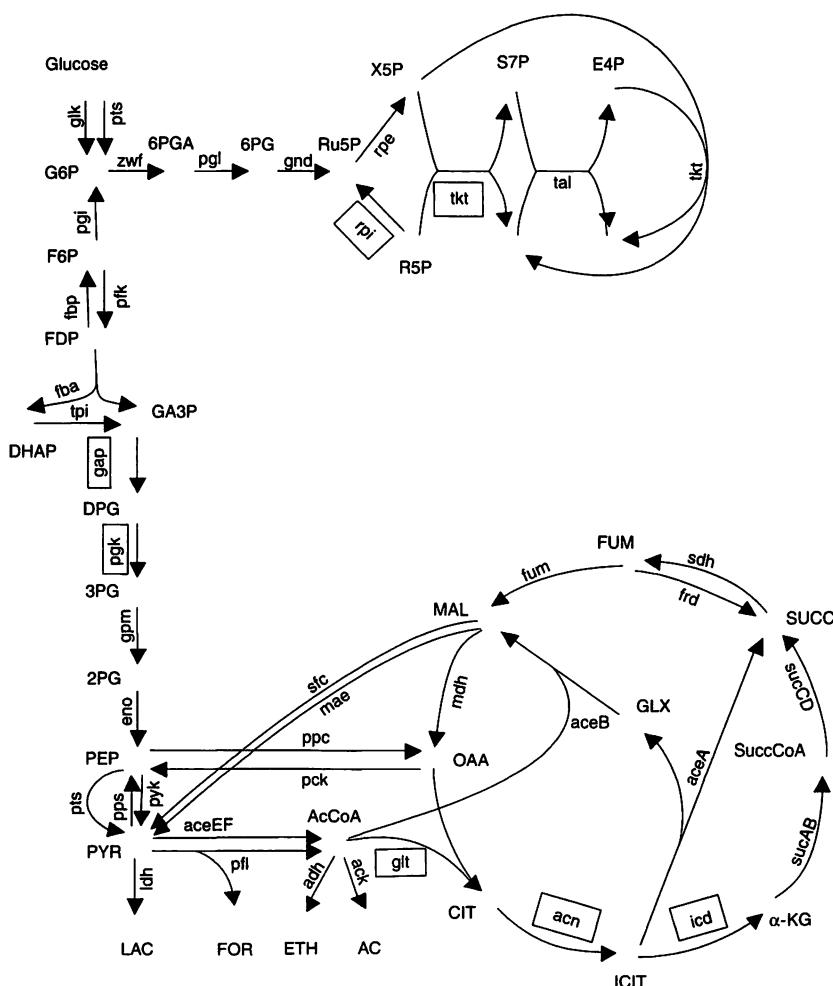
In a series of papers, Edwards and Palsson (131–133) analyzed the robustness of the well-characterized chemical reaction networks in two prokaryotic organisms, *Escherichia coli* and *Haemophilus influenzae*. For *E. coli*, they assembled a reaction network comprising 436 metabolites and 736 reactions from the biochemical literature, genome sequence information, and metabolic databases (132). They then determined the allowable steady-state fluxes under the constraints listed above. Within this allowable region of fluxes, they determined those fluxes for which growth on a minimal medium under aerobic conditions with glucose as sole carbon source was maximal. Growth is maximal for fluxes that produce the necessary metabolites in proportions that correspond to the (empirically known) biomass composition of *E. coli*. (This maximal growth flux distribution can be thought of as a single chemical reaction that converts biosynthetic precursors into biomass with the least possible wastage.) With this optimal flux distribution  $\bar{v}$  in hand, individual fluxes can be forced to a value of zero—corresponding to deletion of the respective enzyme-coding genes—and the effect on growth can then be studied by identifying the highest attainable growth rate with the eliminated enzyme. The parts of metabolism Edwards and Palsson analyzed in this way comprise 48 chemical reactions and include all of glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, and respiration. Intriguingly, only 7 of the 48 eliminated reactions turned out to be essential. That is, their elimination generated fluxes not allowable in

steady state, or fluxes from which an essential biochemical precursor, such as an amino acid, is not produced. Two of the essential reactions, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, are part of the three-carbon stage of glycolysis. Three others are the first reactions of the tricarboxylic acid cycle, namely citrate synthase, aconitase, and isocitrate dehydrogenase. The remaining two essential reactions are part of the pentose phosphate shunt. These reactions are ribose-5-phosphate isomerase and transketolase. All these essential reactions are indicated in an overview of the central metabolic pathways shown in Figure 9.3.

Of the remaining 41 nonessential reactions, 32 reduced cell growth by less than 5%, and only 9 reduced growth by more than 5%. The authors compared their results for a variety of carbon sources to experimental data that had determined the effect of deletions in enzyme-coding genes of *E. coli*. They found that the experimental data and computational predictions on growth/no-growth coincided in 86% of the cases. Thus, the computational results show good agreement with experiment.

The surprising aspect of these results is the high fraction—about two-thirds—of complete loss-of-function mutations that reduce growth by less than 5%. It bears emphasizing that there is no redundancy in this system, in the sense that there are no two enzymes that carry out the same chemical reactions. The explanation of these findings is that flux can be shuttled through parts of a metabolic network that are not affected by blocking a reaction. The following paragraph discusses a concrete example (Figure 9.4) (131) using the enzyme glucose-6-phosphate dehydrogenase, which is part of the pentose phosphate shunt (Figure 9.4). Experimental work shows that loss-of-function mutants in the gene encoding this enzyme grow at near wild-type levels (169).

The pentose phosphate shunt diverts metabolites from glycolysis and serves two main purposes (528). Its oxidative branch generates NADPH for biosyntheses. (This branch can be entered only via glucose-6-phosphate.) Its nonoxidative branch generates biosynthetic precursors such as ribose-5-phosphate. It can be entered from the oxidative branch, but also from other glycolytic intermediates such as fructose-6-phosphate. Edwards and Palsson (131) blocked the first reaction of the pentose phosphate shunt (reaction *zwf* in Figure 9.3), glucose-6-phosphate dehydrogenase, which converts glucose-6-phosphate into D-6-phosphate-glucono- $\delta$ -lactone. This reaction leads into the oxidative branch of the pentose phosphate shunt. Deletion of this reaction completely blocks the oxidative branch but affects the predicted metabolic output only minimally: Wild-type growth is reduced by only 1% under aerobic conditions in glucose minimal medium. However, the deletion has profound systemic consequences on the flux in this network. For example, before the deletion, in the wild-type



**Figure 9.3** Central metabolic pathway reactions in *Escherichia coli*. Reactions in boxes are essential, that is, their removal eliminates growth according to stoichiometric analysis (132, 133). Reactions and genes: *aceA*, isocitrate lyase; *aceB*, malate synthase; *aceEF*, pyruvate dehydrogenase; *ack*, acetate kinase; *acn*, aconitase; *adh*, acetaldehyde dehydrogenase; *eno*, enolase; *fba*, fructose-1,6-bisphosphatase aldolase; *fbp*, fructose-1,6-bisphosphatase; *frd*, fumurate reductase; *fum*, fumarase; *gap*, glyceraldehyde-3-phosphate dehydrogenase; *glk*, glucokinase; *glt*, citrate synthase; *gnd*, 6-phosphogluconate dehydrogenase; *gpm*, phosphoglycerate mutase; *icd*, isocitrate dehydrogenase; *ldh*, lactate dehydrogenase; *mae*, malic enzyme; *mdh*, malate dehydrogenase; *pck*, phosphoenolpyruvate carboxykinase; *pfk*, phosphofructokinase; *pfl*, pyruvate formate lyase; *pgi*, phosphoglucose isomerase; *pgk*, phosphoglycerate kinase; *pgl*, 6-phosphogluconolactonase; *ppc*, phosphoenolpyruvate carboxylase; *pps*, phosphoenolpyruvate

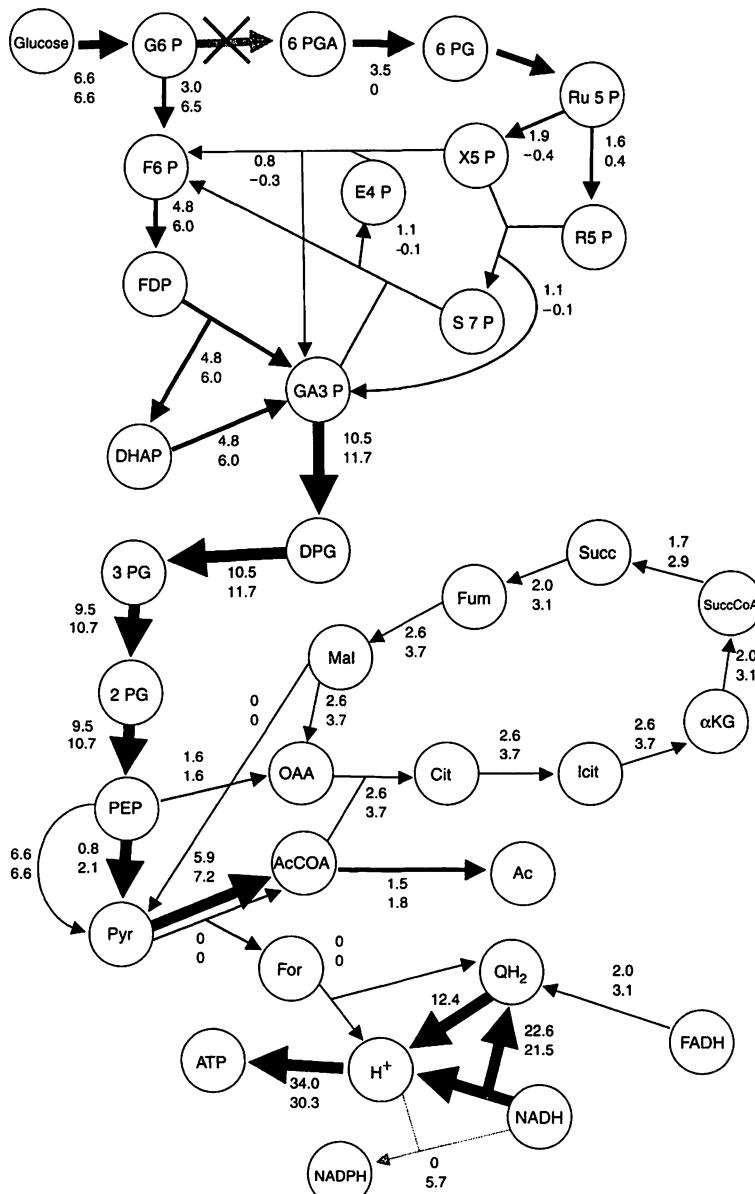
state, about two-thirds of a cell's NADPH is produced by the pentose phosphate shunt. Most of the NADH is produced by the tricarboxylic acid cycle. (High-energy phosphate bonds are largely generated via oxidative phosphorylation.) One of the major systemic reorganizations of metabolic flow after the mutation regards NADPH production. To compensate for the blocked oxidative branch of the pentose phosphate shunt, most of the NADPH is now produced through an increased flux in the tricarboxylic acid cycle, which generates NADH. This NADH is then transformed into NADPH via a massively increased flux through the transhydrogenase reaction. The nonoxidative branch of the pentose phosphate shunt can still be entered through other metabolites of glycolysis, and thus still serves to produce sufficient quantities of biosynthetic precursors. Figure 9.4 shows the fluxes in this mutant.

### Robustness to Flux Reduction Through Essential Reactions

A further striking result of this analysis is that metabolic networks can be robust even to substantial manipulations of flux through essential reactions (133). Specifically, although complete elimination of flux through the seven essential reactions of *E. coli* central metabolism is lethal, substantial quantitative reductions in flux may be neutral. A case in point is the essential transketolase reaction in the pentose phosphate shunt. As long as a mutation preserves more than 15% of the wild-type flux through this reaction, the resulting growth rate is no less than 99.2% of the wild-type growth rate. This absence of a phenotypic effect, however, again camouflages profound systemic changes that have to take place to compensate for the reduction in flux. One of these changes is again an

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synthase; *pts*, phosphotransferase system; *pyk*, pyruvate kinase; *rpe*, ribulose phosphate 3-epimerase; *rpi*, ribose-5-phosphate isomerase; *sdh*, succinate dehydrogenase; *sfc*, malic enzyme; *sucAB*, 2-ketoglutarate dehydrogenase; *sucCD*, succinyl-CoA synthetase; *tal*, transaldolase; *tkt*, transketolase; *tpi*, triosephosphate isomerase; *zwf*, glucose-6-phosphate-1-dehydrogenase. Metabolites: 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; 6PG, D-6-phosphate-gluconate; 6PGA, D-6-phosphate-glucono- $\delta$ -lactone; AC, acetate; AcCoA, Acetyl-CoA; R-KG, R-ketoglutarate; CIT, citrate; DHAP, dihydroxyacetone phosphate; DPG, 1,3-bisphosphoglycerate; E4P, erythrose 4-phosphate; ETH, ethanol; F6P, fructose-6-phosphate; FDP, fructose 1,6-diphosphate; FOR, formate; FUM, fumarate; G6P, glucose 6-phosphate; GA3P, glyceraldehyde 3-phosphate; ICIT, isocitrate; LAC, lactate; MAL, malate; PEP, phosphoenolpyruvate; PYR, pyruvate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; SUCC, succinate; SuccCoA, succinyl CoA; X5P, xylulose 5-phosphate. Modified from Figure 1 of (133). Used with permission.



**Figure 9.4** Rerouting of metabolic fluxes in a metabolic mutant. Growth-maximizing flux for a wild-type metabolic genotype and in the *zwf* mutant. This mutation (indicated by the crossed arrow) eliminates the glucose-6-phosphate dehydrogenase reaction that leads into the pentose phosphate shunt. Predicted biomass yield in this mutant is only one percent lower than that in the wild-type.

increased production of NADPH through the tricarboxylic acid cycle and through transhydrogenase. Another change is an increased flux through glycolytic reactions such as pyruvate kinase that absorb the reduced flux through the pentose phosphate shunt. As flux decreases below 15% through the transketolase reaction, the reduced flux limits the production of erythrose-4-phosphate, an essential precursor of aromatic amino acids. The result is a more severely reduced growth rate.

The first three reactions of the tricarboxylic acid cycle, just as essential as the transketolase reaction, also are quite robust to large quantitative changes in flux. For example, unless flux through the citrate synthase reaction falls below 18% of the wild-type, wild-type growth is essentially unchanged. However, as flux through this reaction is gradually reduced from the optimal wild-type level, a variety of systemic changes occur that allow wild-type growth to be sustained. They include increased flux through the pentose phosphate shunt, reduction and eventual complete elimination of flux through the pyruvate kinase reaction (which is upstream of the blocked reaction), as well as reduction of cyclic flux through the tricarboxylic acid cycle. The cycle eventually ceases to function cyclically, and serves only to generate biosynthetic precursors. If the flux through citrate synthase falls below 18% of wild-type levels, the metabolic network cannot produce sufficient  $\alpha$ -ketoglutarate, an essential precursor of amino acids, to ensure maximal growth. This is the reason for the reduction in growth rate under these conditions (133).

Finally, the two essential glycolytic reactions (glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase) are the most restricted of the seven essential reactions. Their fluxes cannot be reduced to less than 70% of the wild-type level without affecting growth substantially.

I have thus far focused on the effects of mutations that reduce metabolic flux. The majority of mutations will do so, because they reduce either enzyme activity or enzyme expression. However, it is worth mentioning that a metabolic network can also be quite insensitive to *increases* in metabolic flux through individual reactions. For example, flux through the citrate synthase reaction can increase to 160% of wild-type without affecting growth (133).

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Thickness of arrows is proportional to the flux through a reaction in the wild-type. The upper (lower) number next to each reaction indicates the metabolic flux in the wild-type (mutant) [substrate converted  $\text{h}^{-1}$  gram dry weight ( $\text{DW}^{-1}$ )] at a glucose uptake rate of 6.6 mmol glucose  $\text{h}^{-1}$  g  $\text{DW}^{-1}$  and an oxygen uptake rate of 12.4 mmol oxygen  $\text{h}^{-1}$  g  $\text{DW}^{-1}$ . See legend to Figure 9.3 for an explanation of acronyms used. Modified from Figure 3 in (132).

Edwards and Palsson also studied the metabolic reaction network of the bacterium *Haemophilus influenzae* (131). Their results add additional facets to the studies in *E. coli*. The authors used physiological and genome information to construct a map of 488 metabolic reactions and 343 metabolites, and examined robustness of fluxes in this network to deletions in 36 central reactions. They found a larger fraction of essential genes than in *E. coli* (33 vs. 14% in *E. coli*), and a smaller fraction of genes with no effects on growth when eliminated (42 vs. 69% in *E. coli*). However, the networks are not straightforward to compare, because of their different sizes and features. (For example, glutamate was an essential amino acid for the *H. influenzae* network, whereas it can be produced by the *E. coli* network.)

Two aspects of the *H. influenzae* study add substantially to the *E. coli* work. First, the authors here also examined the effects of multiple (double and triple) deletions of enzymatic reactions on growth. They found only 7 lethal double mutants among 361 double mutations whose single mutant constituents were not lethal. Similarly, among 5270 triple mutations, only 7 were lethal. That is, in large metabolic networks under defined conditions, it is quite possible to eliminate multiple network parts without abolishing network function.

The second important aspect of this study is that the authors not only analyzed robustness in one environment, but extended their analysis to several environments. The *H. influenzae* results cited above make specific assumptions about the availability and uptake rate of glutamate, an essential amino acid, and fructose, the sole carbon source. However, the availability of both substrates in the environment may vary widely. When allowing the availability of fructose and glutamate to vary, the number of reactions that did not affect growth when deleted shrank from 14 to 9. When in addition the availability of oxygen was also varied, this number was further reduced to 5. In other words, chemical reactions that do not affect growth in one medium or environment may well do so in another medium or environment. In more general terms, metabolic networks in most living cells need to operate in multiple different environments. Results such as these suggest that most mutations with no effect in one environment may have clear consequences in some other environment (131, 247).

At this point, I must repeat a note of caution to all these results. While the stoichiometric analysis reveals optimality criteria for cell growth, it does not guarantee that cells can attain the requisite fluxes. For example, a cell may not be able to express enzymes in the quantities necessary to ensure maximal growth. In addition, the above predictions of mutational effects on cell growth regard the maximally possible growth flux in a mutant. However, cells may not be able to attain this maximal growth flux, but only a lower, submaximal flux (496). All this applies especially to

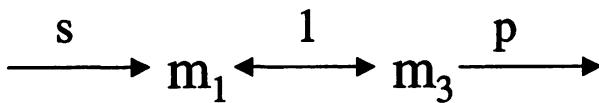
unusual environmental or genetic conditions that a population has not encountered in its evolutionary history, and where no evolutionary pressure has forced an adaptive response. A possible case in point is the *E. coli* double mutant in the enzymes glucose-6-phosphate dehydrogenase and transhydrogenase. Its growth rate is predicted to be 92% of the wild-type (132). However, experimental results from a strain in which both genes encoding the respective enzymes were deleted show that the mutant grows only at 57% of the wild-type rate. This and other quantitative discrepancies may well be due to the fact that *E. coli* strains never have undergone adaptive evolution in the mutant condition (496).

## Robustness and Elementary Flux Modes

The most basic constraint that a metabolic reaction network must fulfill regards the allowed distribution of fluxes in steady state. It is given by equation 9.4 and is a function of the matrix  $S$  of stoichiometric coefficients. The region in the space of all flux vectors that obeys (9.4) is called the null-space of  $S$ . An analysis of its features yields insights into the robustness of metabolic networks beyond the above mutational analysis. Two different approaches are used to analyze the null-space of large chemical reaction networks, elementary flux mode analysis (492, 494) and extreme pathway analysis (430, 431, 606). They are similar in spirit and yield qualitatively similar results. I will focus on one of them here.

A basic and important notion to understand the null-space of a stoichiometry matrix is that of an (elementary) flux mode (492, 494). It is best illustrated with an example. Consider again the simple reaction scheme of Figure 9.1. No mathematical analysis is necessary to appreciate that there are only three basic ways in which a steady-state flux can occur that ensures mass balance for internal metabolites  $m_1$  through  $m_3$  (Figure 9.5). The first leads from external metabolites  $S$  to  $P$  through reaction 1. It can be written as the flux vector  $\bar{x} = (1, 1, 0, 0, 1)$ . The second also leads from  $S$  to  $P$ , but bypasses reaction 1 via the bridge provided by reactions 2 and 3. It corresponds to the flux vector  $\bar{y} = (1, 0, 1, 1, 1)$ . Finally, there is also a cyclic flux that affects neither  $S$  nor  $P$  and involves only reactions 1, 2, and 3. It is characterized by the flux vector  $\bar{z} = (0, -1, 1, 1, 0)$ . The entry of  $(-1)$  in this flux vector reflects that the reversible reaction 1 here produces  $m_1$  from  $m_2$  (and thus proceeds in the reverse direction) to avoid conflict with the irreversibility of reactions 2 and 3.

Any allowable flux through this reaction scheme can be written as a linear combination of these three fluxes, which are also called elementary flux modes. For example, the steady-state flux vector  $\bar{v} = (3, 2, 1, 1, 3)$



$$\vec{x} = (1, 1, 0, 0, 1)$$

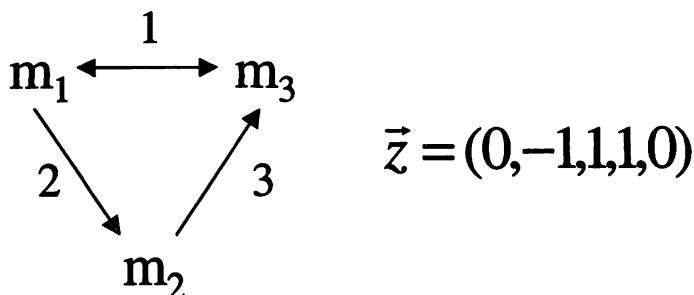
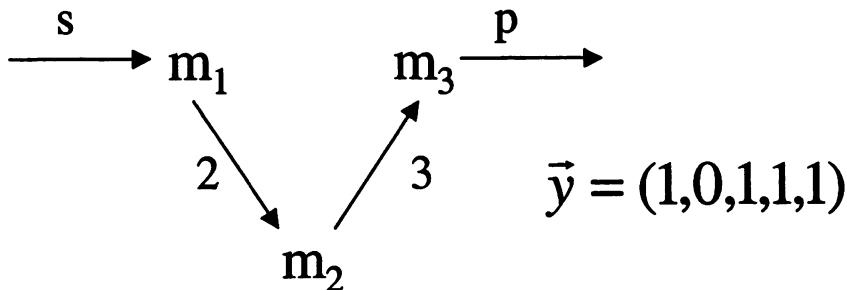


Figure 9.5 Elementary flux modes for the reaction scheme of Figure 9.1.

can be written as  $\vec{v} = 2\vec{x} + \vec{y}$ . While elementary flux modes can thus be thought of as basis vectors into which any allowable flux can be decomposed, two differences to the basis vectors of a vector space in linear algebra are noteworthy. First, the allowable fluxes do not form a vector space, because certain fluxes are prohibited due to constraints such as the irreversibility of some reactions. Second, to facilitate interpretation, one imposes an additional requirement on elementary flux modes. Two elementary

flux modes  $\bar{v}'$  and  $\bar{v}''$  that constitute a flux vector  $\bar{v}$ , e.g.,  $\bar{v} = c_1\bar{v}' + c_2\bar{v}''$ , are not allowed to involve any reactions that are not involved in  $\bar{v}$  itself. For example, the flux mode  $\bar{y}$  in Figure 9.5 is elementary, even though it can be written as  $\bar{y} = \bar{x} + \bar{z}$ . The reason is that both  $\bar{x}$  and  $\bar{z}$  involve reaction 1, whereas  $\bar{y}$  does not. This condition assures that the decomposition of a flux vector into elementary modes involves only those parts of a reaction network through which a flux occurs, and not parts of the network outside the flux vector to be decomposed. In other words, the decomposition of a flux must be a true composition into "subfluxes," which greatly facilitates the biological interpretation of flux modes. More rigorous definitions of an elementary flux mode can be found in (493, 494).

In metabolic networks of realistic complexity, the number of elementary flux modes can be enormous. This is illustrated by the work of Stelling and collaborators (522), who represented central metabolism of *E. coli* as a network involving 89 metabolites and 110 reactions. This representation collapses many of the biosynthetic pathways outside central carbon metabolism into single reactions. However, even with such reduced complexity, the network has more than 27,000 elementary flux modes with glucose as an external carbon source. Only a fraction of these flux modes will allow growth, i.e., will generate all biosynthetic precursors, energy, and redox potential. However, this fraction can still be large. For example, more than 19,000 flux modes support aerobic growth on glucose (albeit some at very small growth rates). The number of growth-supporting flux modes indicates the different ways in which a metabolic network can support growth. This number depends on a variety of factors, such as the available carbon source.

Eliminating a reaction from a metabolic reaction network will reduce the number of elementary flux modes that support growth. As long as one growth-supporting flux mode is left, however, the respective mutation is viable. If no growth-supporting fluxes remain the mutation is lethal. When eliminating individual reactions in *E. coli* central metabolism, more than 40% of the resulting networks still support growth. Comparing the predicted viability of individual mutants with experimental results shows agreement in more than 90% of the cases. The remaining discrepancies result again from the fact that this analysis identifies the possible and not the actually attainable fluxes, because of regulatory and kinetic constraints. In addition, more than two-thirds of the viable mutations reduce the number of growth-supporting flux modes by less than 75% (522). This analysis is purely qualitative, in that a mutant would be classified as viable even if it supported growth at less than 1% of the wild-type. However, it can be extended to determine the fraction of mutants where wild-type growth is reduced by a well-defined amount. As a rule, one finds that mutations that leave more than 50% of flux modes of

the wild-type intact show a growth rate reduction of less than 5%. Even if only 25% of flux modes are left, growth rates decrease by no more than 20% (522). The number of elementary flux modes left after a gene deletion thus serves as a rough correlate of the robustness of growth rates to elimination of enzymes.

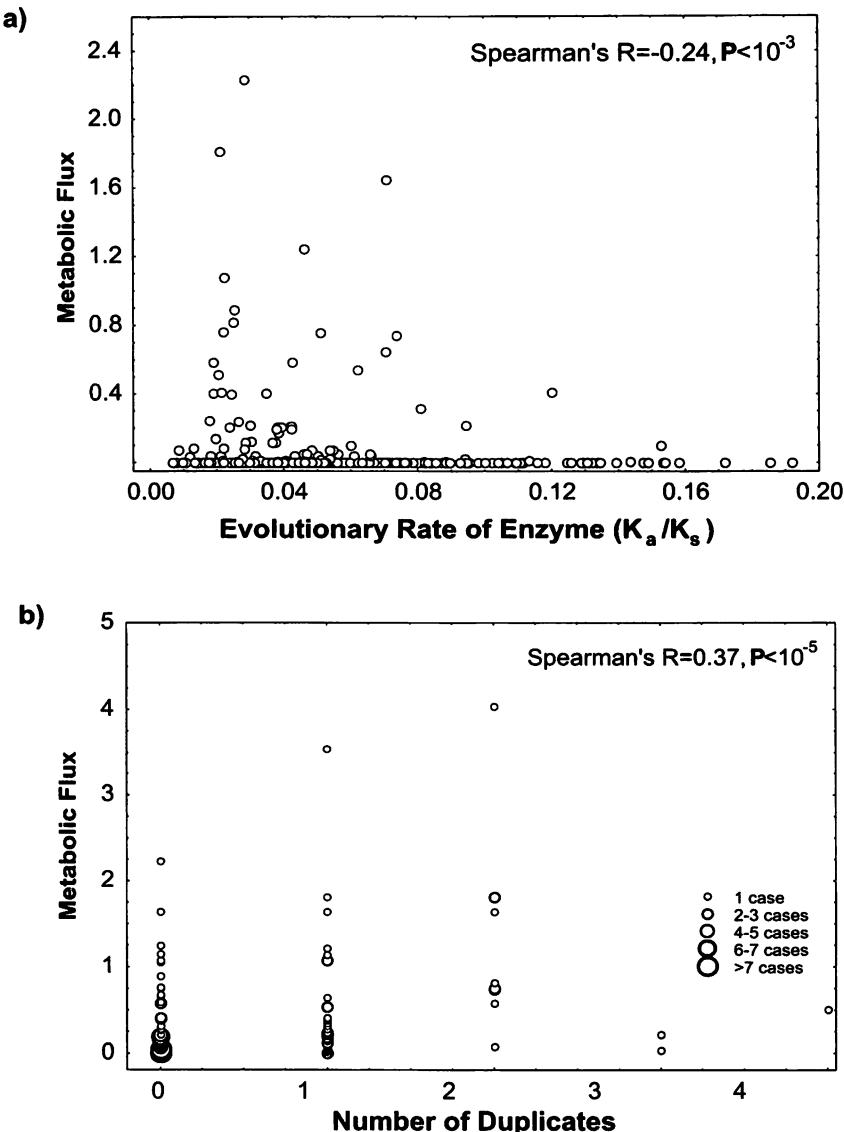
## Robustness and Network Topology

This and the previous chapter show how different representations of a biological system may reveal different sources of robustness. The fine-grained representation of metabolic control analysis incorporates reaction rates of individual enzymes. This representation reveals robustness and distributed control of flux as a consequence of enzyme kinetic laws. Specifically, the activity of any one enzyme may change by a large amount without affecting flux through a pathway. In large metabolic networks, where such fine-grained information is not available, stoichiometric modeling of large genetic networks reveals other facets of metabolic robustness. These include the reorganization of metabolic flux through a large network after individual reactions in the network have been blocked. Can one represent metabolism on an even coarser level and still obtain biologically relevant information on robustness? The next coarser level of representation would eliminate even the stoichiometric information and leave only the bare-bones topology of the network intact. The question how robust networks with different topologies are to well-specified perturbations has received some attention (10, 259, 260). Metabolic networks tell a particularly important—and cautionary—tale, namely that network topology can be misleading when trying to develop biologically meaningful indicators of a network's robustness, as the following paragraphs show.

The coarsest, topological representation of a metabolic network lends itself to one of various graph representations of the network (260, 589). Graphs are mathematical objects that consist of nodes connected by edges. Nodes in this representation correspond to metabolites and/or enzymes. In such a representation of a metabolic network, the distribution of the node degree—the number of neighbors of each node—has a broad tail that is consistent with a power law. In other words, when choosing a node from the network at random, the probability  $P(d)$  that the node has  $d$  neighbors is proportional to  $d^{-\gamma}$ , where  $\gamma$  is a constant characteristic for the network (260, 589). Albert and collaborators (10) found that networks with power-law-distributed degrees are robust to random perturbations in the following sense. Upon removal of randomly chosen nodes, the mean distance between network nodes that can still be reached from each other (via a path of edges) increases only very little. This distance

can be thought of as a measure of how “compact” a graph is. In graphs with other degree distributions, the mean distance between nodes can increase substantially upon node removal. Also, graphs with power-law degree distributions fragment less easily into disconnected subnetworks upon random node removal. These findings have led Jeong and collaborators (260) to suggest that metabolic network graphs with power-law-distributed degrees have such a degree distribution *because* this distribution provides robustness of the network topology against node elimination. In other words, their proposition implies that robustly compact networks confer (unknown) advantages on cells, and that the observed power-law degree distribution is thus a consequence of selection for robust compactness.

While this is an intriguing hypothesis, two lines of evidence speak against it. The first of them relates to the notion that power-law degree distributions of metabolic networks reflect the action of natural selection. If so, then such degree distributions might be restricted to networks potentially shaped by natural selection, or at least to networks with some tangible benefit of robust compactness. Empirical evidence, however, suggests that power-law degree distributions may be a general feature of chemical reaction networks, even networks that have never been under the influence of natural selection to begin with. For instance, Gleiss and collaborators (192) have assembled publicly available information on a class of large chemical reaction networks that exist not only outside the living, but on spatial scales many orders of magnitude larger than organisms. These are the chemical reaction networks of planetary atmospheres, networks whose structure is determined by the photochemical properties of atoms and molecules in a planet’s atmosphere. The available data stem not only from earth’s atmosphere, but also from other solar planets, including Venus and Jupiter, planets with chemically vastly different atmospheres. These atmospheres have been explored through remote spectroscopic sensing methods or through visits by planetary probes. The chemical reaction networks in these atmospheres have a degree distribution consistent with a power law (192), despite being made up of very different chemical compounds. This suggests that power-law distributions may be very general features of chemical reaction networks in both living and nonliving systems. Even more generally, networks—both natural and human-made—with power-law degree distribution are literally everywhere. They occur in biology, physics, chemistry, engineering, linguistics, and sociology (9). This suggests that the power-law degree distribution is perhaps a principle of network organization as fundamental as the ubiquity of Gaussian distributions in nature. It may teach us very little about biology and even less about mutational robustness resulting from natural selection favoring robustness.



**Figure 9.6** (a) Yeast metabolic enzymes that carry high metabolic flux evolve more slowly (568). The horizontal axis shows the rate of enzyme evolution, as estimated by the ratio  $K_a/K_s$  of amino acid replacement to silent nucleotide substitutions that occurred in enzyme-coding genes of the yeast *Saccharomyces cerevisiae* (336). The vertical axis shows metabolic fluxes (millimoles of substrate converted per gram dry weight and per hour) through individual enzymatic reactions. The ratio  $K_a/K_s$  of *S. cerevisiae* genes is estimated relative to their unambiguous orthologues

The second line of evidence stems from a corollary of the hypothesis that power laws reflect evolved robustness of a network. The compactness of networks with broad-tailed degree distributions, although insensitive to removal of randomly chosen nodes, is highly sensitive to targeted removal of highly connected nodes. The greater a node's degree, the more likely it is that the node's removal decreases the network's compactness or causes network fragmentation. This means that in a representation of a metabolic network where nodes correspond to enzyme-coding genes, and where two enzymes are connected if their reactions share at least one metabolite, the network's integrity should be more vulnerable to perturbations of highly connected enzymes than of lowly connected enzymes. If this hypothesis is correct, then cell growth should be more susceptible to mutations in highly connected enzymes, and highly connected enzymes should evolve more slowly, because they can tolerate fewer mutations. The reason would be that mutations in highly connected enzymes are more likely to have drastic effects on the network's topology.

In the yeast *Saccharomyces cerevisiae*, highly connected enzymes do indeed evolve more slowly (568). Specifically, nucleotide substitutions that cause amino acid changes accumulate more slowly in genes encoding highly connected enzymes. The crucial question is whether the drastic effects of highly connected enzymes on network topology are the root cause of this phenomenon. The answer is probably no. To see this, consider the periphery of a metabolic network, part of which consists of linear metabolic pathways that are fed by the products of metabolism's densely interconnected core. Any one such linear pathway may produce essential compounds such as amino acids or biosynthetic cofactors. Blocking it may thus be fatal. However, enzymes in such peripheral, linear pathways are by definition not highly connected. Thus, elimination of these enzymes would not change the network's topology dramatically, yet the enzymes may be essential (568). These simple considerations raise the question of what, if not network topology, is responsible for the slow evolution of some enzymes? Put differently, what biological features of an enzyme can explain its rate of evolution better than an enzyme's effect on network topology? A possible answer is shown in Figure 9.6. Much

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in three *Saccharomyces* species with completely sequenced genomes (276). Metabolic fluxes are shown as predicted by flux balance analysis (166) of yeast cells growing in aerobic conditions on minimal medium with glucose as sole carbon source. (b) Enzyme-coding genes with high associated flux have more duplicates (568). The analysis includes only genes with fewer than five duplicates and enzymes with metabolic flux  $F < 5$ , which constitute the vast majority of all genes and enzymes.

like in a highway network, the flux of matter through reactions in central metabolism—which feeds multiple peripheral pathways—may be much greater than the flux at the periphery (13). Central metabolism may thus be more sensitive to amino acid changes in individual enzymes, most of which reduce but do not completely eliminate flux (13). Figure 9.6a shows the relation between metabolic flux through an enzyme and the rate at which the enzyme evolves through amino acid changes: Exactly as one would predict, the higher the metabolic flux through an enzyme, the lower its rate of evolution. The following observation underscores the biological significance of this finding, the association between evolutionary rate and metabolic flux. Gene duplications are mutations whose effect on enzymatic reactions is the opposite of amino acid changes, most of which reduce metabolic flux. That is, gene duplications create two or more copies of an enzyme-coding gene and may thus facilitate the flux of matter through any one enzymatic reaction. Thus, the benefits of retaining gene duplicates might be higher for enzyme-coding genes whose products carry high metabolic flux. Figure 9.6b shows that this is indeed the case: Enzyme-coding genes whose products carry high metabolic flux tend to have more duplicates (247, 568).

Taken together, these observations suggest that network topology does not provide all biologically relevant information on mutational robustness in metabolic networks. A minimal yet useful representation needs to include other information, such as the distribution of metabolic fluxes following from stoichiometric information. This further underscores a point I made earlier: How we choose to represent a biological system determines what we can learn about its robustness.

In sum, a stoichiometric representation of metabolic networks shows that such networks can be highly robust to elimination of individual chemical reactions or genes, and to the variation of metabolic flux through such reactions. Metabolic networks achieve such robustness through a systemic reorganization of the flux of matter through the network, a reorganization which ensures that the network continues to produce biosynthetic building blocks and energy carriers. In this sense, metabolic networks have a large associated neutral space (chapter 13) of alternative flux patterns, patterns that ensure continued maximal cell growth. Importantly, however, mutations that do not affect cell growth—or other results of metabolic outputs—in one environment may well do so in other environments.

# 10

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## *Drosophila* Segmentation and Other Gene Regulatory Networks

What is robust? The expression pattern of *Drosophila* segment polarity genes, and gene expression patterns in other gene regulatory networks.

What is it robust to? Changes in regulatory interactions among network genes, in gene copy number, and in the expression patterns of genes “upstream” of the network.

This chapter illustrates how one can make educated guesses about the robustness of a genetic network, even when hobbled by incomplete information. The chapter uses one main example (571), the network responsible for subdividing the *Drosophila* embryo into multiple segments. This example comes from a growing body of work on quantitative models of gene regulation networks, models that are firmly grounded in empirical information (14, 23, 51, 136, 142, 334, 369, 370, 387, 458, 497, 560). Such models integrate an enormous volume of data into a mathematical network representation. The large amounts of necessary data—usually accumulated by hundreds of researchers—raises two problems. First, the same data can motivate models with subtle differences, differences that are impossible to evaluate for all but the few experts familiar with all relevant evidence (11, 387, 571). In addition, the experimental information motivating some models, the model’s mathematical details, and an exhaustive discussion of its behavior might by itself fill a book. For this reason, I chose to focus here on only one maximally illustrative example network and to give an overview of relevant information. I chose this network because its robustness has been studied most explicitly and because the approach to study its robustness is pioneering. In addition, other studies hint at similar features in different networks (14, 32, 142, 455), such that this example may stand for a much broader class of gene regulation networks.

## Background on Fly Segmentation

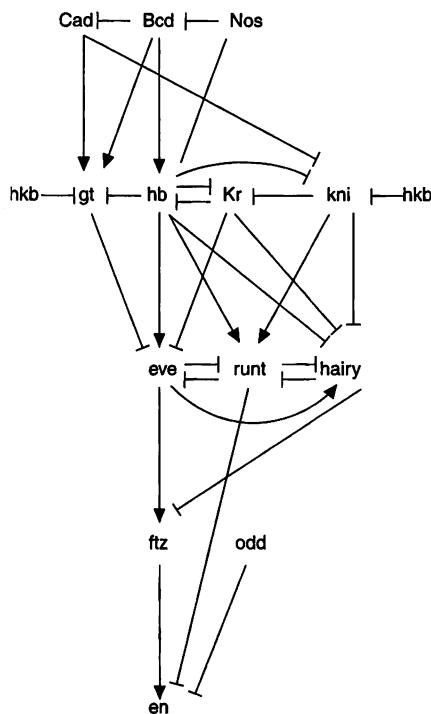
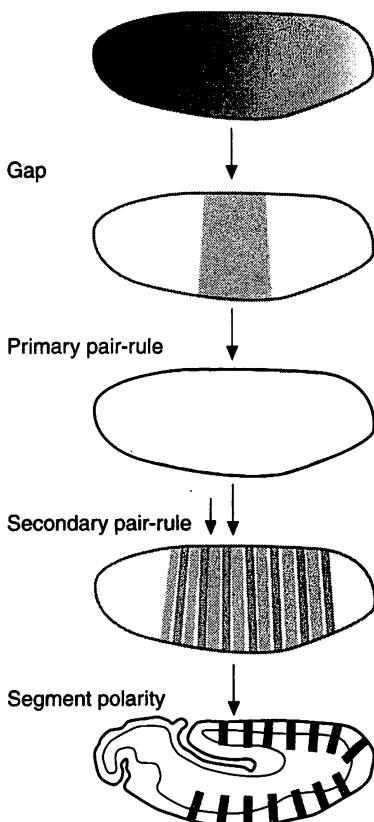
The *Drosophila* segmentation network is one of the best-studied networks in animal development, a network that has seen many thousand man-years of intense experimental analysis. I now briefly describe its inner workings. More comprehensive accounts can be found in developmental biology texts (72, 189).

Within merely 24 hours, a fertilized *Drosophila* egg develops into a highly organized, motile feeding larva with a sophisticated nervous system. One of the hallmarks of this larva, and of insects in general, is its subdivision into more than a dozen distinct segments. The network of genes responsible for segmental patterning is well studied. It consists of five different classes of genes (Figure 10.1), genes that can be distinguished by the time at which their characteristic expression patterns are established.

The earliest expressed genes are the maternal genes, whose mRNAs the mother—hence the name—deposits in the fertilized egg, where they are translated. These genes include *bicoid*, *caudal*, and *nanos*, whose protein products are expressed in broad anterioposterior gradients in the egg. These gradients are not identical. For example, the Bicoid protein has its highest concentration at the anterior pole of the egg, whereas the caudal protein is most abundant at the posterior pole. Two main mechanisms generate these gradients. The first is translational regulation. Bicoid, to give one example, inhibits the translation of the *caudal* mRNA, which is responsible for caudal's gradient being inverse to that of Bicoid. The second mechanism is free diffusion of proteins, made possible by the fact that the early embryo is a syncytium—it lacks cell walls. For example, the Bicoid gradient arises from Bicoid's diffusion from the anterior to the posterior pole. The lack of cell walls has another important consequence: it eliminates the need for elaborate cell-to-cell signaling. Free diffusion of early segmentation gene products is sufficient for the earliest embryonic pattern formation events. Partly for this reason, most of the early segmentation genes encode transcriptional regulators instead of cell signaling molecules.

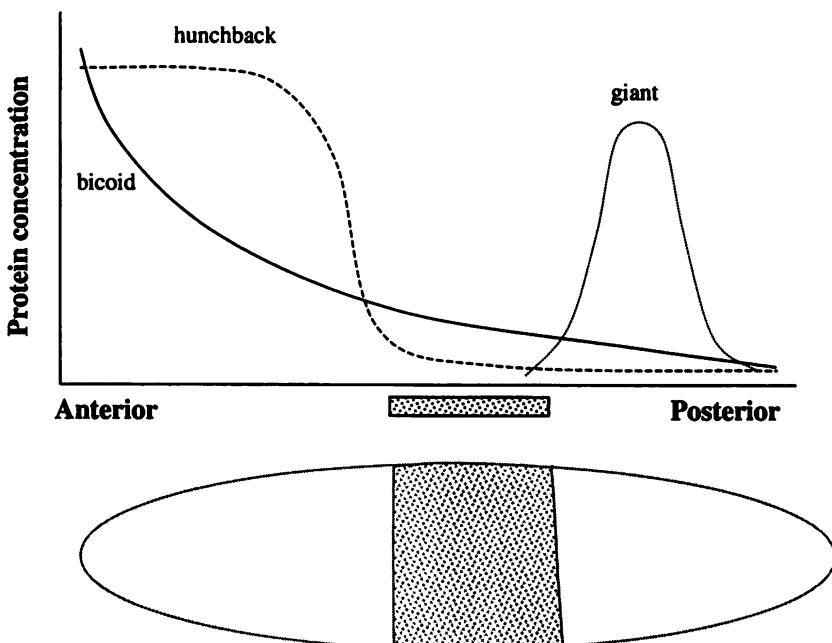
The next class of genes, gap genes, are not expressed in broad gradients, but in discrete contiguous regions within the embryo. Loss-of-function mutations in these genes lead to embryos that lack large contiguous sections—gaps—which correspond to the gap gene expression domains. Both maternal gene expression gradients and cross-regulatory interactions among gap genes are necessary for gap gene expression. For example (Figure 10.2), the gap gene *Krüppel* is expressed in a broad region in the prospective thorax. Its expression is activated by the maternal gene product Bicoid. However, Bicoid's activation is overridden by repression through Hunchback in the anterior part of the embryo and by repression

## Maternal



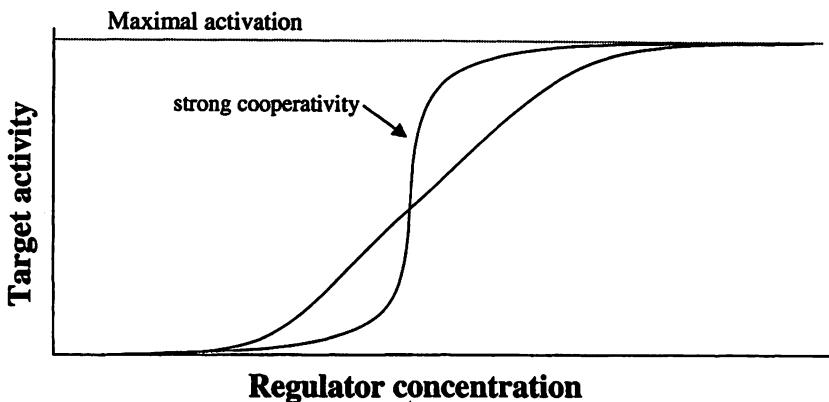
**Figure 10.1** Selected members of five gene classes in the early segmentation of the *Drosophila* embryo, and their regulatory interactions. An arrow indicates a positive regulatory interaction, whereas a cross-bar at the end of a line indicates a negative, repressive interaction. Maternal genes include *caudal* (*Cad*), *bicoid* (*Bcd*), and *nanos* (*Nos*). Gap genes include *hunchback* (*hb*), *giant* (*gt*), *Krüppel* (*kr*), *knirps* (*kni*), and *huckebein* (*hkb*). Primary and secondary pair rule genes include *even-skipped* (*eve*), *runt*, *hairy*, *fushi tarazu* (*ftz*), and *odd-skipped* (*odd*). Only one among several segment polarity genes, *engrailed* (*en*), is shown. Although most of these proteins are transcriptional regulators, not all of their regulatory interactions occur on the level of transcription. From Figure 3.5 in (72).

through the gap protein Giant in the posterior part. All these regulatory interactions are transcriptional. That is, the regulatory proteins bind enhancer regions near the *Krüppel* gene to regulate its transcription. Similarly complex interactions determine the expression of other gap genes (Figure 10.1).



**Figure 10.2** A representative example of some of the regulatory interactions that establish gap gene expression. The oval below the graph is a schematic view of the *Drosophila* embryo (anterior = left, dorsal = up). The anterior boundary of the expression domain (in gray) of the gap gene *Krüppel* is determined by transcriptional repression through Hunchback, which overrides transcriptional activation by Bicoid. Its posterior boundary is determined by repression through other regulators including Knirps and Giant.

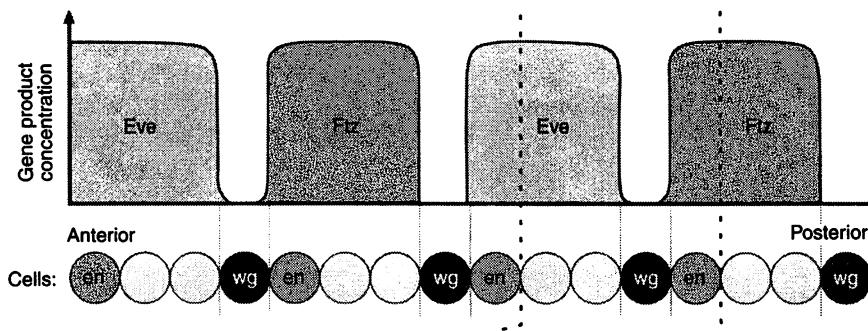
A frequent feature of transcriptional regulation among early segmentation genes, and among many other genes, is cooperativity (Figure 10.3). For instance, a maternal regulatory gene product such as Bicoid can bind the regulatory DNA region of a gap gene, such as *hunchback*, at multiple different binding sites. Binding to one of the sites facilitates binding to other sites—hence the name cooperativity. This cooperativity has an important consequence. As the concentration of the regulator increases, the transition between a state where no binding site is occupied (the regulated gene is not expressed) and one where all sites are being occupied (the regulated gene is maximally expressed) is reached at a sharp threshold. Such thresholds, caused by cooperative binding, can lead to steep expression gradients of some genes, even though their regulators are expressed in shallow gradients. (Figure 10.2 shows the shallow gradient of



**Figure 10.3** Cooperativity in the regulation of biomolecule activity. The activity of a “target” molecule (vertical axis) is influenced by the concentration of a regulatory molecule (horizontal axis). As the concentration of the regulator increases, the target activity increases until it reaches some maximal value. With strong cooperativity, the transition between minimal and close to maximal target activation is achieved over a small range of regulator concentrations. (An extreme case is switch-like target activation.) The degree of cooperativity can be measured by cooperativity coefficients, such as the Hill coefficient (150), which is the slope of the relationship between regulator concentration and target activity at the point of half-maximal target activation. Cooperativity is abundant both in transcriptional regulation and in ligand–receptor interactions.

Bicoid, and the much steeper gradient for hunchback, which is mediated by cooperative transcriptional regulation through Bicoid.)

After gap genes, the next two classes of genes are primary and secondary pair rule genes, including *fushi tarazu*, *even-skipped*, and *odd-skipped*. They are expressed in seven narrow stripes, whose elimination generates embryos that lack every other segment. The expression pattern of primary pair rule genes is driven largely by transcriptional regulation through maternal and gap genes, which is no less complex than the regulation establishing gap gene expression. Secondary pair rule genes, in turn, establish their seven striped expression patterns by combinatorial activation and repression through primary pair rule genes. It is important to note that the seven stripes of pair rule expression are not congruent for all pair rule genes, but that they are displaced relative to each other. This displacement allows effective combinatorial expression regulation of the fifth and last class of genes, segment polarity genes. Segment polarity genes, the most prominent of which are *engrailed*, *wingless*, and *hedgehog*, are expressed in 14 stripes whose numbers correspond to the larval segments. Again, these stripes are not congruent. For example, *wingless*



**Figure 10.4** Expression of segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) in cells (circles) along the anterior–posterior axis of the *Drosophila* embryo. The establishment of *en* and *wg* expression depends on transcriptional regulation by pair rule genes such as *ftz* and *eve*, whose expression domain is indicated. The boundary between *en* and *wg* expressing cells defines the boundary of 14 parasegments. That is, *wg* is expressed at the posterior boundary of each parasegment, whereas *en* is expressed at its anterior boundary. Only four parasegments are shown. The boundaries of segments are displaced one cell to the right of the parasegment boundaries, and are indicated for one segment by two vertical dashed lines. From Figure 14.25A in (189).

is expressed in a stripe of cells adjacent to a stripe of cells expressing both *engrailed* and *hedgehog*. The boundaries between these cell stripes do not delineate the segments themselves, but are displaced anteriorly toward the middle of each segment. That is, they delineate the boundaries of so-called parasegments (Figure 10.4). Segment polarity gene expression is initially established, again, through transcriptional regulation, regulation carried out by pair rule genes. For example (Figure 10.4), the expression of *wingless* is activated by transcriptional regulators ubiquitous in the embryo, but is repressed wherever pair rule genes *fushi tarazu* and *even-skipped* are expressed. The net result is that *wingless* is expressed only in the narrow stripe of cells where both these regulators have low concentrations (Figure 10.4). Conversely, *engrailed* is expressed in cells adjacent to *wingless* expressing cells. Its expression is driven by transcriptional activation through *fushi tarazu*, and lack of repression by another pair rule gene, *odd-skipped*.

By the time segment polarity gene expression becomes established, cell walls have formed in the embryo. Free diffusion of most regulators throughout the embryo is no longer possible. Cell signaling thus gains increasing importance in embryonic patterning. In consequence, although some segment polarity genes, such as *engrailed* and *cubitus interruptus* encode transcriptional regulators, many others encode cell signaling molecules. For instance, Wingless and Hedgehog are each secreted proteins

that act as ligands in cell-cell communication. *Patched*, another segment polarity gene, encodes a transmembrane protein that is a receptor for Hedgehog.

The seven striped expression patterns of pair rule genes are ephemeral. They disappear after segment polarity gene expression is established. However, continued segment polarity gene expression is necessary for proper segmental patterning. This expression must be maintained by different means, for which interactions between segment polarity genes are crucial. Specifically, cells that express *wingless* secrete the Wingless protein, which binds to a receptor on *engrailed*-expressing cells immediately posteriorly. This receptor activates a signal transduction pathway whose end point is a transcription factor that activates and maintains *engrailed* expression. Engrailed, in turn, activates the transcription of the *hedgehog* gene. Hedgehog is secreted and binds to its receptor, Patched, on adjacent *wingless* expressing cells. This causes derepression of the transcription factor Cubitus interruptus, which can then activates *wingless* expression. The net result is a regulatory cycle. *Wingless* expressing cells are necessary for continuing *engrailed* expression in adjacent cells, and *engrailed* expressing cells are necessary for continued *wingless* expression.

### A Model of the Segment Polarity Gene Network

The gene expression cascade culminating in segment polarity gene expression is perhaps the best understood developmental process of any organism. However, this understanding is largely qualitative. That is, the genes involved in regulating the activity of most genes in the cascade are known, as are some general principles of this regulation. For example, it is well established that transcriptional regulation through competing transcription factors (Figure 10.2) is paramount for the regulation of early segmentation genes, and that much of this regulation is cooperative. Unfortunately, quantitative data about regulatory interactions is unavailable for most genes. Affinities of transcription factors to their binding sites, the extent of their cooperative binding, absolute concentrations of protein products, the rates at which signaling molecules diffuse through the embryo, their affinities to membrane receptors, these and many other quantitative characteristics of the segmentation network are poorly understood. In the few instances where such quantities have been measured, measurement errors are large. This problem is by no means typical for *Drosophila* segmentation or developmental processes. It holds also for other genetic networks that are exceptionally well characterized, such as the network responsible for bacterial chemotaxis (24), or the network driving the bacteriophage lambda from a dormant, lysogenic state to a

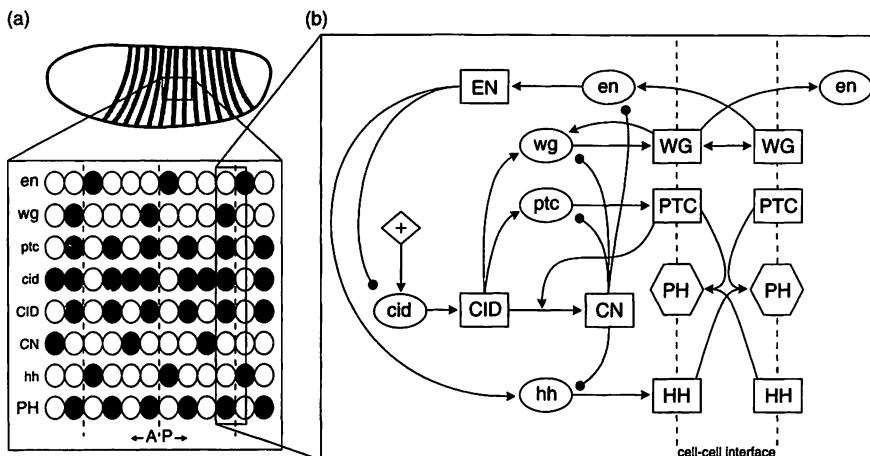
virulent, lytic state (449). Part of the reason is that there are many more relevant parameters than genes, and measuring each one of them is just as laborious—and less glamorous—than characterizing the genes in the first place.

Because of this lack of quantitative information, an obvious approach to study network robustness is blocked. This approach would consist of producing a quantitative model of the network and of calculating how changes in key genes affect network function. Absent such a model, it would also not be useful to perturb the network systematically through mutations and to assess how its behavior changes. This approach—immensely useful to identifying network genes in the first place—would show only that some mutations affect the network whereas others do not.

However, a variant of this approach proposed by von Dassow and collaborators (571) may provide hope—perhaps the only hope—for this and other well-characterized networks. These investigators restricted themselves to analyzing the robustness of segment polarity gene expression in *Drosophila*. They first established a minimal model of segment polarity gene regulation based on empirically observed interactions among segment polarity genes. This model includes the interactions I briefly discussed above in the maintenance of *engrailed* and *wingless* expression (Figure 10.5). In the model, concentrations and activities of gene products can change over time due to regulatory interactions among network genes. These regulatory interactions are defined by 48 free (and mostly unknown) parameters that describe the rate of transcription, translation, chemical stability (decay) of gene products, degree of cooperativity in transcriptional regulation, strength of transcriptional activation or repression as a function of regulator concentration, diffusibility of signaling molecules, and binding constants to their receptors.

The model sketched in Figure 10.5a is by no means complete, and it rests on empirical observations with varying degrees of support. For example, the autoregulation of *wingless* occurs through a poorly characterized mechanism, and the regulation of *engrailed* expression by *wingless* is the result of a complex signal transduction pathway that involves its receptor Frizzled, several other signaling molecules, such as Disheveled, Zeste-white 3, and a terminal transcription factor, Armadillo, which directly activates *engrailed* expression. The model collapses all these steps into one regulatory interaction. Because of these simplifications, it is best to think of the model as a prototype that captures qualitatively important features of the segment polarity network.

Von Dassow and collaborators asked an important question in relation to the robustness of this network (571): What parameter combinations—what regions in the 48-dimensional parameter space—yield a functioning network? More specifically, once expression patterns of *engrailed* and



**Figure 10.5** A model for maintenance of segment polarity gene expression. (a) The upper panel shows a schematic representation of a *Drosophila* embryo with expression stripes of Wingless and Engrailed along parasegment boundaries. The lower panel shows the expression patterns of these and multiple other segment polarity genes in multiple cells (ovals) for cells spanning more than two parasegments. Black ovals indicate cells where the respective gene is expressed. (b) The part of the segment polarity network modeled by von Dassow and collaborators (571). Lowercase acronyms correspond to genes, uppercase acronyms to their products; +, basal, unregulated expression. Arrows indicate either gene expression or positive regulatory interactions, lines terminating in circles repressive interactions. Although shown only for one cell and one cell-to-cell interface, each cell of the embryo contains one instance of this network. Its components vary in activity across cells.

The model incorporates the following qualitative observations from the experimental literature. The transcription factor Engrailed (en) represses the expression of *cubitus interruptus* (cid), and activates the expression of *hedgehog* (hh), whose product is secreted, and diffuses to adjacent cells, where it binds its receptor, Patched (PTC) to form the complex indicated as PH in the figure. This causes a reduction in the rate at which Cubitus interruptus (CID) protein is proteolyzed to form the transcriptional repressor CN. The full-length gene product (CID) is an activator of *wingless* (wg) and *patched* (ptc) expression, whereas the proteolyzed fragment (CN) inhibits *wingless*, *patched*, and possibly also *engrailed* expression (572). *Wingless* expression leads to Wingless secretion and to eventual transcriptional activation of *engrailed* in neighboring cells. *Wingless* is also autoregulated. The Cubitus interruptus gene product (full-length or proteolyzed) inhibits Hedgehog activity. After Figure 5 from von Dassow (572).

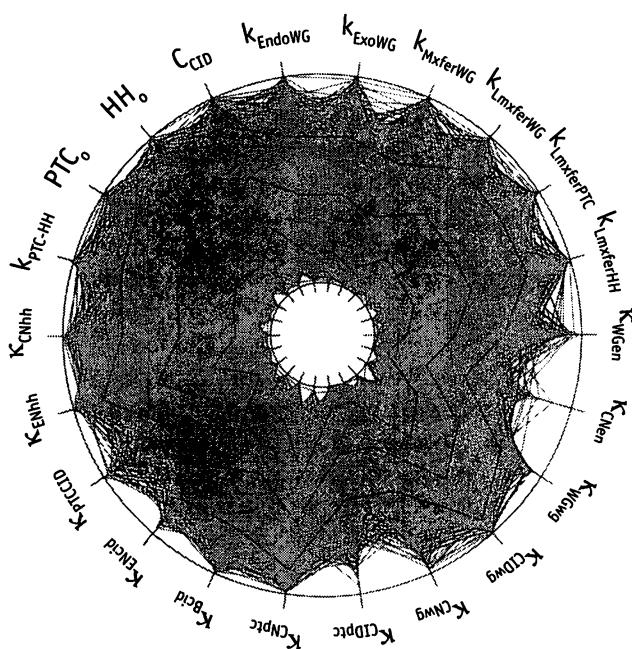
*wingless* are established by pair rule genes upstream of the network (Figure 10.1), which parameter combinations allow the network to sustain the empirically observed gene expression patterns schematized in Figure 10.5a? They addressed this question simply by first sampling parameter combinations at random from this high-dimensional parameter space, and then asking whether the resulting segment polarity network is functional. That is, for any one such parameter combination, does the network arrive at the observed segment polarity gene expression pattern?

Note that this approach does not identify whether the actual segment polarity network is robust to perturbations. It could not do so, because the segment polarity network is a point of in this high-dimensional parameter space whose exact location is unknown. Instead, the approach crudely characterizes the structure of the entire parameter space for all networks whose qualitative pattern of interactions—the topology shown in Figure 10.5a—is identical to the segment polarity network. If one finds preciously few parameter combinations that yield functional networks, networks that reproduce the wild-type gene expression pattern, then the robustness of networks with the given topology is poor.

### Robustness of the Segment Polarity Network

Each of the model's 48 parameters can be thought of as one dimension or axis of a 48-dimensional space. This space is bounded because each parameter is restricted to biochemically realistic values that span two to three orders of magnitude (571, 572). Put differently, the set of biochemically possible parameter values is a rectangle in a 48-dimensional space. The many dimensions are crucial in making the case for the network's robustness. Assume, for the purpose of illustration, that for any one of the 48 parameters, 10% or a fraction  $f = 1/10$  of values within the parameter's biologically meaningful range are found in functional networks. In this case, functional networks would inhabit only a vanishingly small fraction  $F = (1/10)^{48}$  of the parameter space. More generally, if picking a parameter value at random has a probability  $f$  of success, meaning that the parameter value is allowed in a functional network, the fraction  $F$  of the space occupied by functional networks decreases exponentially with the dimension of the space ( $F = f^n$ ).

In the network model of Figure 10.5, one in 200 randomly chosen parameter combinations yields a functioning network, i.e.,  $F = 1/200$ . From this value, one can estimate the fraction  $f$  of each parameter's values that would yield a working network as  $e^{\ln F/n} = e^{\ln(1/200)/48} = 0.895$ . In other words, almost 90% of any one randomly chosen parameter value would



**Figure 10.6** Wheel plot of parameter combinations that yield a functional segment polarity network. Each spoke of the wheel represents one parameter (only 22 out of 48 parameters are shown), and each position along one spoke represents one value within the allowable range (typically three orders of magnitude) for the parameter. Gray polygons correspond to parameter combinations that yield functional networks. Note that for any one spoke, parameter values that yield functional networks are distributed over a wide part of the allowed range. Black polygons represent the mean (middle)  $\pm$  one standard deviation (outer and inner) of such parameter values. Parameters labeled  $k_{X,Y}$  correspond to the concentration of regulator X at which it affects the activity of Y at its half-maximal rate;  $X_0$  is the maximally possible steady state concentration of gene product X;  $k_{PTC-HH}$  is the (second order) rate at which PTC and HH bind to each other;  $k_{endoX}$  and  $k_{exoX}$  correspond to rates at which X enters and leaves a cell through endocytosis and exocytosis;  $k_{MxferX}$  and  $k_{LmxferX}$  are the rates at which X is transferred between adjacent and apposite cell faces;  $C_{CID}$  is the maximum cleavage rate of protein product CID. (See Figure 10.5 for explanations of other symbols.) From Figure 2a in (571) by permission of the Nature Publishing Group.

yield a working network. The space of parameters is thus very densely populated with working networks, as shown in a wheel-plot representation of allowable parameter combinations (Figure 10.6). This high value of  $f$  is even more striking if one considers that individual parameter values are allowed to span a two order-of-magnitude range.

A complementary approach to assess network robustness builds on the ease of choosing functional networks in the parameter space. This approach departs from one randomly chosen parameter combination that yields the wild-type, experimentally observed gene expression pattern. One then varies the value of one parameter while holding all others constant, and asks whether the network keeps maintaining segment polarity gene expression. The answer is no less striking. While there are a few functional networks for which some parameters may vary only twofold, in many others most parameters may vary across half their range or more without affecting network function (572). Network behavior is extremely robust to variation in individual parameters.

One can also ask how variation in network topology affects the fraction of parameter space occupied by functioning networks (571, 572). Why ask this question? First, because mutations could alter network topology. Second, because the network model is incomplete. That is, the actual network's topology may differ slightly from that of the network model. Most variants of the topology in Figure 10.5 that produce the correct gene expression pattern occupy similarly large fractions of parameter space. However, for a small subset of topological variants, more or fewer working parameter sets exist (572). For example, in the model of Figure 10.5, two forms of the transcriptional regulator *Cubitus interruptus* affect the expression of the *wingless*. A full-length form activates *wingless* expression, whereas a proteolyzed form represses *wingless* expression. Because both of these forms may bind similar regulatory DNA sequences, they may also compete with each other's binding sites. If one postulates that the proteolyzed form (CN in Figure 10.5) inhibits the activation of Wingless by the full-length form, the fraction of parameter space with working solutions decreases by more than a factor 10 to  $F = 1/2000$ . This illustrates the importance of network topology. Not all network topologies are equally likely to accommodate functional networks.

Changes in interaction parameters or network topology are not the only source of network variation. Another source is changing concentrations in the five gene products that form the network. Such changes can be caused by the environment—either outside or inside the embryo—or by genetic changes in gene copy numbers. Gene copy number can change in two ways, the first being gene duplications, the second loss of function of one gene (heterozygous null mutations). The segment polarity network is also quite robust to such changes. Depending on the gene whose dosage changed, between 30 and 80% of the networks from Figure 10.5 continued to function in a heterozygous null genotype. In addition, between 30 and 97% of networks can tolerate up to 6 extra copies of any one of the five modeled network genes gene. The frequency of networks in the parameter space that could tolerate both heterozygosity and up to

6 extra copies of *each* gene was  $F = 5 \times 10^{-5}$  (572). This implies that more than 80% of random parameter choices yield a functional network robust to drastic changes in gene dosage.

A final indicator of robustness regards variation in the *engrailed* and *wingless* expression patterns. These patterns activate the segment polarity network, and one can ask how much deviation in them the network can tolerate and still maintain segment polarity gene expression. Variation in the expression pattern of these genes could again be caused by mutations in pair rule genes, by mutations in other segmentation genes, or by environmental variation, such as variation in embryo size (243, 517). The network is quite robust also to this kind of variation. Even if *engrailed* and *wingless* are expressed not in sharply demarcated expression stripes but in shallow gradients that extend over multiple cells, resembling only faintly the sharply periodic wild-type expression pattern, one in 5000 networks, or 83.7% of randomly chosen parameter combinations, yield a functional network. In addition, for some initial *engrailed* and *wingless* expression patterns that are not periodic at all, the parameter space contains many networks (one in 300–600 networks; 87.5–88.8% of parameter values) that arrive at the proper segment polarity expression pattern as long as some other network genes show periodic expression (571).

In sum, a very large fraction of randomly chosen parameter combinations render the segment polarity gene network of Figure 10.5 functional. Vary any one of these parameters over a wide range, and network function may change very little. Most network variants with different topology occupy a similarly large fraction of parameter space. In addition, networks robust to large increases or decreases in gene copy number or to variation in the “input” expression pattern also occupy a large fraction of parameter space.

Again, these results have to be consumed with caution. Most importantly, the model of Figure 10.5 is incomplete and—like any model—uses a much simplified representation of known facts. Additional segment polarity genes exist; gene expression patterns other than those of *engrailed* and *wingless* are necessary for their establishment; the embryo is not an array of hexagonal cells; its cells proliferate and become rearranged during development, and so on. However, the model contains many essential interactions, and many of its topological variants perform similarly well, which calls for cautious optimism about its relevance. In addition, an extension of the model based on more recent and extensive empirical evidence underscores the network’s robustness to mutations (251a).

In more general terms, the approach pursued by von Dassow and collaborators points toward an avenue of assessing robustness in networks where interactions are qualitatively well understood but quantitatively

poorly understood. (All cellular and developmental networks characterized to date fall in this category.) However, the approach addresses two questions central to this book only incompletely. The first is this: How robust is network performance to genetic change? A very large fraction of parameter space is occupied by functional networks, which suggests that many random mutations would be neutral in their effect on network function. However, this inference is suggestive, not conclusive. Unfortunately, not even the observation that network function is robust to changes in individual parameters answers this question. One reason is that most mutations—in either gene coding or regulatory regions—may change more than one parameter simultaneously. For example, to mutate a binding site for a transcription factor may change both its degree of cooperativity and the maximal rate at which it can activate its target gene; a mutation in a gene's coding region can change both a protein's half-life and its affinity to a receptor, and so on. Another reason is that genetic networks or parts thereof are often used in multiple different circumstances. For instance, segment polarity genes are also used in the development of wings and eyes. Such additional functions can further constrain—in unknown ways—the regulatory interactions necessary for a properly functioning network.

The second open question is this: If the segment polarity network is robust to the effects of mutations, how did it come to be that way? Did it evolve such robustness—and if so, through what evolutionary force—or is such robustness intrinsic to any network of similar topology? Parameter space is rife with functional networks, which suggests that robustness might be an intrinsic feature of any network with the topology of Figure 10.5a. However, the structure of this parameter space need not be uniform. That is, some parts of the space may contain many more functional networks than other. By the evolutionary mechanisms I outline in Chapter 16 and 17, a genetic network can come to reside in regions of parameter space enriched with functional networks, regions where any one mutation has a high chance of being neutral. Unfortunately, we do not know whether such evolution of robustness has occurred for this network, or for other intensely studied and robust gene regulation networks. However, the following section suggests that the segment polarity network may belong to a much larger class of networks that readily give rise to great mutational robustness, and where such robustness is further evolvable.

### Minimal yet Robust Networks

Faced with incomplete information, the model of Figure 10.5 incorporates only general assumptions about the segment polarity network's regulatory interactions. Nonetheless, many variants of the network produce proper

segment polarity gene expression. These observations raise the possibility that robustness may characterize a universe of networks that share some very general features. What could these features be? The most obvious candidates are cooperative interactions among regulatory molecules (Figure 10.3). Cooperativity is a common feature of regulatory processes such as transcriptional regulation and ligand-receptor interactions (61, 261, 290, 444, 525, 595). The stronger a cooperative interaction, the less the activity of a regulated molecule will change over a broad range of regulator concentrations. Cooperativity can therefore be viewed as an instance of robustness to changing regulator concentrations. What effects does such robustness have on whole regulatory networks?

The following is a minimal model of a network ideally suited to address this question. It contains an extreme form of cooperativity, namely switch-like behavior of individual genes (579). The individual genes can be thought of as encoding transcriptional regulators that mutually influence each other's expression. Such transcriptional regulation networks are not only important in fly development. They also occur in the development of many other many-celled organisms and in single-celled organisms (325). A case in point are the transcriptional networks formed by homeobox genes, which are central to patterning of body axes in most animals (189).

Specifically, the network model involves  $N$  genes that can assume different expression states in one cell during embryonic development. (Because most development requires signaling across cells, the action of a network consisting exclusively of transcriptional regulators is best modeled inside one cell only.) The expression state of a gene  $i$  at some time  $t$  during development is represented by a variable  $S_i(t)$  that can assume only two values, "on"  $S_i(t) = (+1)$  or "off"  $S_i(t) = (-1)$ .  $S_i(t)$  can be potentially influenced by the expression of another gene  $S_j(t)$  via a regulatory interaction represented by a real-valued variable  $w_{ij}$ . If  $w_{ij} > 0$ , then  $j$  is a transcriptional activator of  $i$ , whereas if  $w_{ij} < 0$ , then  $j$  is a repressor of  $i$ . Some or most values  $w_{ij}$  may be different from zero, meaning that any one gene's expression may be influenced only by a subset (a proportion  $c$ ,  $0 < c < 1$ ) of other network genes. In the network as a whole, the expression state (on or off) of any gene  $i$  can change over time due to influences of other network genes. With switch-like cooperativity, this change is best described by the following mathematical expression:

$$S_i(t + \tau) = \sigma \left( \sum_{j=1}^N w_{ij} S_j(t) \right) \quad 1 \leq i \leq N \quad (10.1)$$

where  $\sigma(x) = (+1)$  if  $x > 0$  and  $\sigma(x) = (-1)$  if  $x < 0$ . Time is represented as discrete steps of length  $\tau$ , which can be thought of as a characteristic timescale of transcriptional regulation.

An extreme form of cooperativity, switch-like behavior, permeates this model. A gene is expressed only if the number of its expressed activators exceeds a threshold and the number of its expressed repressors falls below a threshold. The threshold is determined by the strengths of regulatory interactions  $w_{ij}$ . The model is clearly a bare-bones, highly abstract representation of a class of network involving cooperativity. However, this very class of models has also proven very successful in modeling the transcriptional regulation network within early fruit fly development and in predicting both regulatory interactions and mutant phenotypes (458, 498). Its origins are in computational neuroscience (229).

For a network (10.1) that is “primed” with some initial gene expression pattern (analogous to the initial *engrailed* and *wingless* expression pattern) and that arrives—through regulatory interactions—at some stable and unchanging expression pattern, one can ask the following question about robustness. How sensitive is the network’s stable expression pattern to mutations in regulatory interactions (corresponding to random changes in one of the parameters  $w_{ij}$ )? The answer is that at least 60% of such regulatory changes, where a value  $w_{ij}$  is replaced with a random number chosen from some prespecified distribution, do not affect the stable gene expression pattern at all (579). This high fraction of neutral mutations is independent of network size ( $4 < N < 10$ ), of the number of regulatory interactions in the network ( $0.4 < c < 1$ ), and of the distribution of the regulatory interactions in the network (579).

With this network model, one can also ask whether robustness to mutations in regulatory interactions is an evolvable feature. The results I just summarized were based on networks with randomly chosen parameters that arrive at a specified gene expression pattern—analogous to the approach taken in the segment polarity network model. One can subject such networks to stabilizing selection, selection favoring networks that produce a stable expression pattern. This is best done by simulating network evolution in a population of organisms where each organism harbors one copy of the network. The population undergoes mutation (in regulatory interactions  $w_{ij}$ ), recombination among network genes, and natural selection to maintain the stable gene expression pattern of the network. Through the mechanism I discuss in greater detail in Chapter 16, this process weeds out networks whose expression patterns are sensitive to mutations. At the end of several hundred generations of such simulated evolution, one finds that the robustness of networks to random changes in regulatory interactions has increased from 60% to between 85 and 95% (579). Smaller networks and networks with fewer regulatory interactions increase

robustness to a lesser extent than larger networks with many interactions. The actual gene expression pattern of network genes is unimportant, as long as some stable gene expression pattern is favored (499). In sum, gene regulation networks that incorporate cooperative regulatory interactions show robustness to mutations that can change through evolution by natural selection. However, this does not mean that cooperativity is the only general principle underlying network robustness. There may be many other such principles, such as an abundance of feedback cycles (251a) whose importance for robustness is easy to appreciate, but also principles that remain to be discovered.

## Outlook

Analyses of gene regulation networks are a flourishing industry and I have only scratched the surface here with two examples. These examples represent two among multiple complementary ways to represent biological networks, such as discrete switching networks, networks continuous in space or time, stochastic networks, Boolean networks, and so on. While existing experimental and theoretical work on regulatory networks could fill many books, the analysis of network robustness has, with some exceptions, been only an afterthought and not the main focus of analysis. I chose the above examples because they address the problem of robustness most explicitly and clearly. The examples also best illustrate a strategy to cope with our ignorance about details of gene interactions within such networks: Make minimal assumptions consistent with empirical evidence and analyze the behavior of whole classes of networks that meet these assumptions. The examples do so in different ways. The first assumes a network topology that is based on empirical information, whereas the second example is even more abstract, assuming only that networks show cooperative regulatory interactions. The strategy they represent will certainly not become less important in the future. Although regulatory networks become increasingly better characterized, such characterization also yields insights into fundamental uncertainties that will not disappear with better data. For instance, it is increasingly appreciated that the numbers of regulatory molecules in a cell fluctuate randomly. Such fluctuations can critically influence a regulator's behavior, as well as the behavior of a network composed of such regulators (49, 138, 366, 454, 544, 545).

To summarize, regulatory networks with many cooperative gene interactions show robustness to mutations. The segment polarity gene network is a specific example of such a network, and part of the mechanistic cause for its robustness may be the many cooperative interactions of its genes.

Robustness can increase in evolution, if the network's gene expression pattern is under the influence of selection favoring the persistence of this pattern. This suggests that the high-dimensional space of possible parameters in such networks—the 48 parameters of the segment polarity network and the strengths of the many possible regulatory interactions in the abstract model—is not homogeneous. That is, the space may have regions where most mutations have no effect on network function, and other regions where networks are less robust and exceedingly fragile.

# 11

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## Phenotypic Traits, Cryptic Variation, and Human Diseases

What is robust? The developmental pathways that form phenotypic characters, and the characters themselves.  
What is it robust to? Variation in the genes of these pathways.

This chapter focuses on robustness in the developmental processes that produce macroscopic characters such as eyes and wings. Specifically, it discusses evidence that many phenotypic characters vary little, despite much variation in the genes involved in their development. Because such cryptic variation is the result of past mutations, this phenomenon shows that developmental pathways and the characters they produce are robust to mutations. I first review some of the historically earliest experiments demonstrating widespread mutational robustness in embryonic development. I then discuss more recent experimental work on the striking role of one specific gene, the gene encoding the heat shock protein Hsp90 in such robustness. Finally, I argue that Hsp90 is only an extreme example of a general phenomenon, namely that a gene's effect on the organism is influenced by other genes. For illustration, I discuss a body of literature on the genetics of human diseases. This literature shows that robustness to disease-causing mutations is itself genetically controlled. Robustness as an organismal trait thus meets one of the key prerequisites that makes a trait's evolution through natural selection possible.

### Detecting Robustness by Breaking It

Often, large numbers of controlled genetic perturbations—necessary to evaluate a system's robustness—are difficult to make if not outright infeasible. This holds especially for the embryonic development of macroscopic traits in many-celled organisms, because development is controlled by genetic systems that comprise hundreds of genes, systems

that are incompletely characterized. In this case, however, one can still resort to compare equivalent solutions that related species have found to one and the same biological problem. The work on enhancer evolution from chapter 7 illustrated this approach and its main disadvantage: One can study only a small number of alternative solutions to the problem.

Some studies in this chapter—variants of this comparative approach—alleviate this shortcoming. That is, these studies compare *many* different solutions to the key problem of embryonic development: how to reliably build organismal features such as wings, eyes, or legs. These studies do so by comparing many different individuals of one and the same population. Put differently, the studies I discuss compare naturally occurring variation in genes responsible for organismal development. However, while their approach circumvents one problem of comparative analyses, it has other problems. Specifically, the genetic variation it analyzes is not controlled; yes, the genes in which this variation occurs are usually not even known.

The studies I review first take a circuitous route to detect robustness. Specifically, they show that development is robust to ubiquitous genetic variation, but not by detecting robustness under normal conditions. No, they generate conditions—such as mutants in individual genes—under which such robustness breaks down. By doing so, they also show that robustness can be genetically controlled, which makes its change in biological evolution possible.

## Early Experiments

At a time where targeted mutagenesis of a genetic system was impossible—the structure of DNA had not even been unveiled—the first genetic experiments already demonstrated robustness to naturally occurring genetic variation. Thus, although the following examples tell us little about the mechanisms underlying such robustness, they are important historically: They were the first systematic demonstrations of genetic robustness. Scharloo presents a detailed review of these early experiments (483).

Among the earliest work, the experiments of the embryologist Waddington stand out. He first demonstrated that robustness of organismal development, a phenomenon he termed canalization (578), is under genetic control. In one set of experiments, he studied abnormalities in fruit fly development. Fruit fly wings have a characteristic pattern of veins that crisscross the wing and provide it with structural stability (Figure 11.1) (515). Very few flies in wild populations show abnormal wing veins. Waddington (575) found that a particular type of wing abnormality vastly

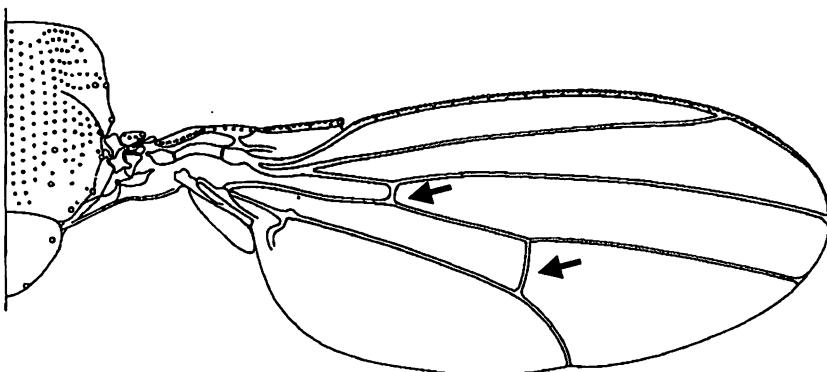


Figure 11.1 Dorsal view of a wing of the fruit fly *Drosophila melanogaster*. The arrows indicate the position of two cross-veins. One or both of these cross-veins were interrupted or disappeared altogether in the experiments of Waddington (575). Image from Flybase (546) (<http://flybase.org/>).

increases in frequency if developing flies are subject to high temperatures (40°C) for several hours, a so-called heat-shock treatment. This abnormality is the absence of one or both cross-veins between two longitudinal wing veins (Figure 11.1). This finding, in and by itself, was not surprising. It was already known at the time that extreme physiological or environmental conditions can influence organismal development (195, 317). The surprise came with the next experiments (575), for which Waddington first established two populations of flies in which developing flies were subjected to a heat shock. In one of these populations, he selected only flies for reproduction that had shown the *cross-veinless* phenotype after the heat shock. In the other population, he selected flies that had not shown the *cross-veinless* phenotype. After a dozen generations of alternating heat shocks and selective breeding, the incidence of flies that reacted to the heat shock changed in both lineages. Specifically, after a heat shock, more and more flies developed abnormal cross-veins in the lineage selected for flies with such abnormalities. In contrast, fewer and fewer flies developed such abnormalities in response to the heat shock in the other lineage. After merely 12 generations, the frequency of flies with cross-vein abnormalities differed by 55% in the two lineages. Waddington also reared a fraction of flies in each of the two lineages without any heat-shock treatment. He found that an increasing number of flies in the lineage selected for increasing sensitivity to heat shock were *cross-veinless* without any heat-shock treatment.

These experiments show that the flies with and without cross-veins after heat shock differ genetically. Otherwise, artificial selection could not have

produced flies that are *cross-veinless* in the absence of the heat shock. Experiments in which the initial population was highly inbred support this hypothesis (34). Such a population contains little or no genetic variation as a result of inbreeding, and shows no change in the incidence of *cross-veinless* flies after selection. In addition, experiments like that of Waddington suggest that genetic variation in wing development is hidden in the starting population (before the onset of artificial selection). This variation does not become manifest under normal environmental conditions, but only under heat shock. Put differently, the structure of wings is robust to variation in genes influencing its development. This robustness breaks down under heat treatment, thus exposing cryptic genetic variation.

Waddington demonstrated the same phenomenon with the more striking *bithorax* phenotype (576, 577). In normal flies, only the second segment on the thorax carries wings. The third carries halteres, a pair of organs necessary for balance during flight. In *bithorax* flies, the third segment resembles the second segment, and, in extreme cases, carries a second pair of wings instead of the halteres. Adult flies form the *bithorax* phenotype in response to ether exposure during early development. In selection experiments analogous to those for the *cross-veinless* phenotype, Waddington showed that the frequency of the *bithorax* phenotype can increase over multiple generations. Like *cross-veinless*, the *bithorax* phenotype eventually arises in the absence of ether treatment.

In an important variation on this theme, other groups of researchers (124, 125, 459, 460, 483) studied unusual phenotypes that arise not through chemical treatment or temperature changes, but through genetic changes. The first notable examples of this sort are the experiments by Dun and Fraser (124, 125) on the number of secondary vibrissae in mice. Vibrissae in mice are important for orientation, surface discrimination, and balance. Wild-type mice have 19 secondary vibrissae that are found under the chin, on the left and right side of the facial part of the head, and on each forelimb. This number is nearly invariant, differing only in 0.2% of wild-type mice. However, this picture fundamentally changes if the *Tabby* (*Ta*) mutation in the sex-linked gene *Eda* (encoding the ectodysplasin-A protein) is introduced into a population. This gene is involved in the formation of structures that derive from the epidermis, such as sweat glands, teeth, hair—and vibrissae. In populations where *Tabby* segregates, secondary vibrissae number varies widely among individuals. Also, the mean number of vibrissae is reduced to 15 for *Ta/+* females and 8 in *Ta/Ta* females and *Ta/Y* males. In two different populations containing the *Tabby* mutation, Dun and Fraser selected for increasing and decreasing vibrissae numbers. After 19 generations, *Ta/+* individuals in the two lineages differed by an average of 8 vibrissae. Variation in vibrissae number remained high in the selected populations.

In a similar line of experiments, Rendel (459) studied developmental variation in scutellar bristles of the fruit fly. The scutellum is a shield-like region on the dorsal part of the thorax, which normally has several bristles, sensory organs that serve as touch sensors. Wild-type flies have 4 scutellar bristles, two of which occur anteriorly, and the other two posteriorly on the scutellum. Their number and arrangement is so invariant that it characterizes the entire genus *Drosophila*. Rendel introduced a mutation in the *scute* gene into a wild-type fly populations with invariant bristle numbers. *Scute* is a sex-linked gene that encodes a transcription factor involved in sex-determination, nervous system formation, and bristle development. In fly populations where the *scute* mutation segregated, bristle numbers began to vary widely from zero to four bristles, with a mean of one bristle in males and two bristles in females. In artificial selection experiments, Rendel succeeded in increasing the number of bristles, such that the mean number of bristles in *sc/sc* flies approached the original four bristles in the wild-type (+/+) genotype. In such a lineage selected for increased bristle number, however, wild-type (+/+) flies have up to five and six bristles. Similar to Waddington's earlier experiments, these experiments show that populations harbor cryptic genetic variation that can be brought out under suitable conditions. Importantly, by using a genetic perturbation—the introduction of the *scute* mutation—to break robustness, they show that not only the environment but also individual genes may influence this variation. Unfortunately, it is not known what genes harbor this cryptic variation, nor is it known how robustness to such variation is normally achieved or how this robustness disappears in the presence of alleles such as *Tabby* and *scute*. Thus, our ignorance about the genetic mechanisms behind such robustness is nearly complete, with few exceptions (126, 187, 452, 471). The most striking such exception regards mutations in a well-characterized gene that abolish robustness of a variety of fruit fly characters and developmental processes.

## Hsp90 and Fly Development

The heat-shock protein 90 is encoded by the gene *Hsp90*, a member of a very large family of related genes. Collectively known as heat-shock genes, the family members occur in both eukaryotes and prokaryotes. They are essential to the survival of all organisms (200, 309). Originally named heat-shock genes because they become activated in response to sudden and large temperature increases, they may help cells cope with a much wider variety of environmental stressors, such as energy depletion or the presence of reactive oxygen species. Many family members also act in un-stressed cells, where they are essential in helping proteins fold properly.

Specifically, they prevent aggregation of nascent proteins into large, misfolded complexes, and they restore misfolded protein folding intermediates to their native configuration. In other words, they are necessary for maturation and maintenance of a functioning proteome. They preferentially bind to patches of hydrophobic amino acids on a protein's surface, patches that in many correctly folded proteins are buried deep in the protein's interior. Such hydrophobic patches may serve as a signature of protein misfolding, and thus trigger refolding by a mechanism that is still incompletely understood.

Like other heat-shock proteins, Hsp90 is essential in eukaryotes, abundant in cells, and activated by stress. However, Hsp90 is unusual among heat-shock proteins because it is not required for maturation and maintenance of most proteins under normal conditions. Instead, many of its target proteins belong to a class of proteins with specific functions: signal transduction—the transmission of molecular messages across cells. At least 80 different proteins involved in a great variety of signal-transduction pathways are bound by Hsp90 (447). By binding to Hsp90, these proteins remain poised for activation until the molecular signal activating them occurs. Signal transduction is essential for the development of any many-celled organism. The reason is that development of even the simplest phenotypic feature requires hundreds or thousands of cells to divide and differentiate in a precisely choreographed fashion. The importance of Hsp90 for many proteins in signal transduction renders development vulnerable to changes in Hsp90 function.

Mutations eliminating the fruit fly *Hsp90* gene are lethal in homozygotes and can only be maintained as heterozygotes. Rutherford and Lindquist (471) detected an unusually high incidence (1–5%) of morphological abnormalities in fruit fly strains into which they had introduced *Hsp90* mutant alleles by genetic crossing. This increased incidence of abnormalities occurred both in multiple wild and laboratory populations, and was further aggravated by extreme temperatures, consistent with Hsp90's role as a protector against thermal stress. The kind of abnormalities that occurred depended on the strain into which Hsp90 was introduced. What distinguishes these abnormalities from those detected in the older experiments I described above is their broader spectrum of phenotypes. Aside from abnormalities in wing shape, wing venation as well as bristle numbers, multiple other abnormalities occurred. They included abnormal eye structure, deformed legs and halteres, abdominal deformities, and transformations of thorax segments. Some of these abnormalities—missing eyes, small wings, and extra antennae—were severe. Virtually any macroscopic trait can be affected by *Hsp90* mutations. This is consistent with the importance of Hsp90 for multiple signal transduction pathways, and for multiple different developmental processes.

Several lines of evidence show that these abnormalities were due to Hsp90 malfunction caused by mutations in the *Hsp90* gene (471). First, different mutations, all of which affected Hsp90, showed a similar spectrum of abnormalities within the same fly strain. Second, treatment of flies with a specific inhibitor of Hsp90, the drug geldanamycin, also yielded a similar spectrum of abnormalities. Third, the same morphological defects occurred in F1 siblings of crosses between *Hsp90* mutant strains and other laboratory strains. In contrast, different F1 families, where the parents carried different *Hsp90* mutations, showed different defects. This suggests that the shared defects come from shared genes in the parental mating pair. A similar pattern was observed when F1 flies with morphological defects were crossed: The F2 generation of one mating pair shared morphological defects. In a fourth series of experiments, Rutherford and Lindquist generated artificial selection in two fly lineages, each bearing a different morphological defect, a deformed eye (Figure 11.2) and thickened wing veins. For each of these phenotypes, they generated six artificial selection lines. In three of these lines they selected for flies with abnormalities (high expression lines) over multiple generations of artificial selection. In the other three lines they selected against flies with abnormalities (low expression line). Within less than 10 generations, more than 50% of flies in three replicate high-expression lines expressed the abnormal phenotype, as shown in Figure 11.3 for the deformed eye phenotype. In addition, a spectrum of different abnormalities arose in both the deformed eye and wing vein lines. Rutherford and Lindquist subsequently also showed that both the deformed eye and the wing vein abnormalities showed positive heritability, further bolstering the case for genetic determination of these abnormalities.

As in the earlier experiments, the appearance of the abnormalities eventually became independent from the presence of the *Hsp90* allele. That is, after more than 15 generations of artificial selection, none of the individuals with either wing venation or eye deformities carried the original *Hsp90* mutation that had elicited the phenotype.

### Heat-shock Proteins and Robustness in Other Organisms

The ability of Hsp90 to provide robustness of phenotypic characters against genetic variation is not a peculiarity of fruit fly development. It also occurs in the flowering plant *Arabidopsis thaliana* (452). This sessile, autotrophic organism is about as different from the vagile, heterotrophic fruit fly as an organism can be. In addition, its populations also show a very different pattern of genetic variation. A typical fruit fly population contains large amounts of genetic variation, whereas a typical

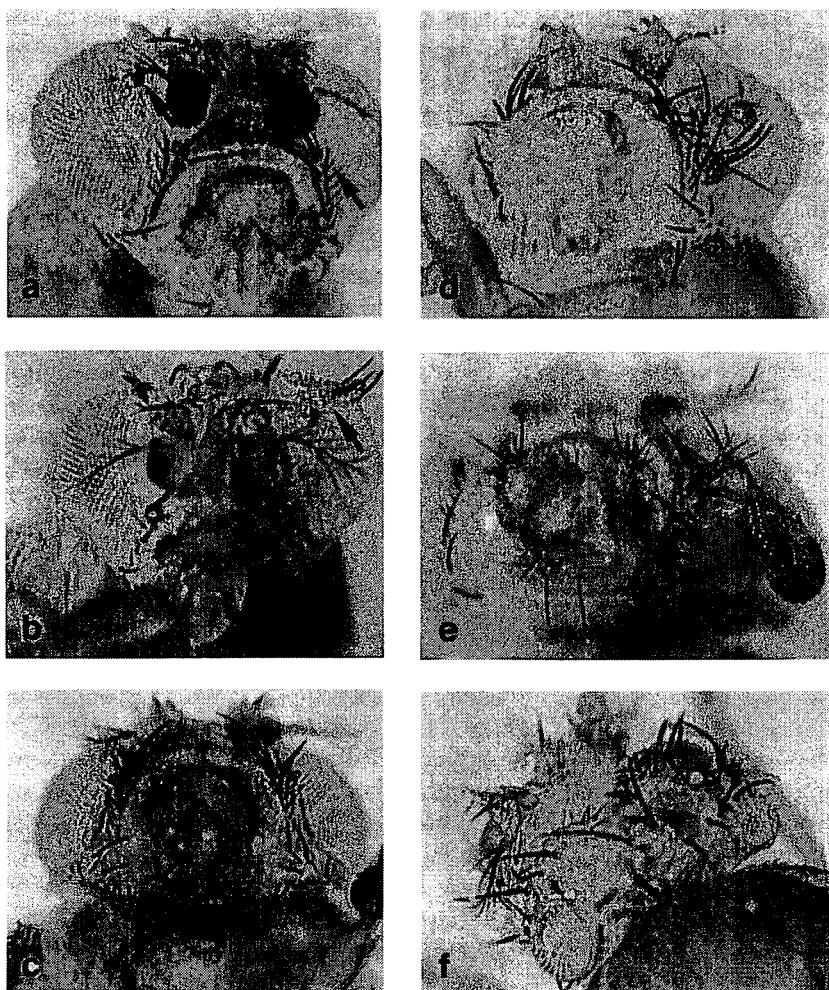


Figure 11.2 Individuals from three fruit fly populations in which Rutherford and Lindquist (471) selected for increased incidence of eye deformities that originally occurred after introducing a *Hsp90* mutation into the populations. (a) Very mild duplications of bristles (arrows); (b) small protuberances (arrow); (c–e) severe bristle duplications and deformities; (f) A severely affected animal. From Figure 3 in (471) by permission of Nature Publishing Group.

*Arabidopsis* population is highly inbred and contains very little genetic variation (5, 315). Different inbred *Arabidopsis* populations differ genetically but are genetically homogeneous within the population.

Queitsch and collaborators (452) inhibited the action of Hsp90 in *Arabidopsis* populations with two different and specific inhibitors of Hsp90,

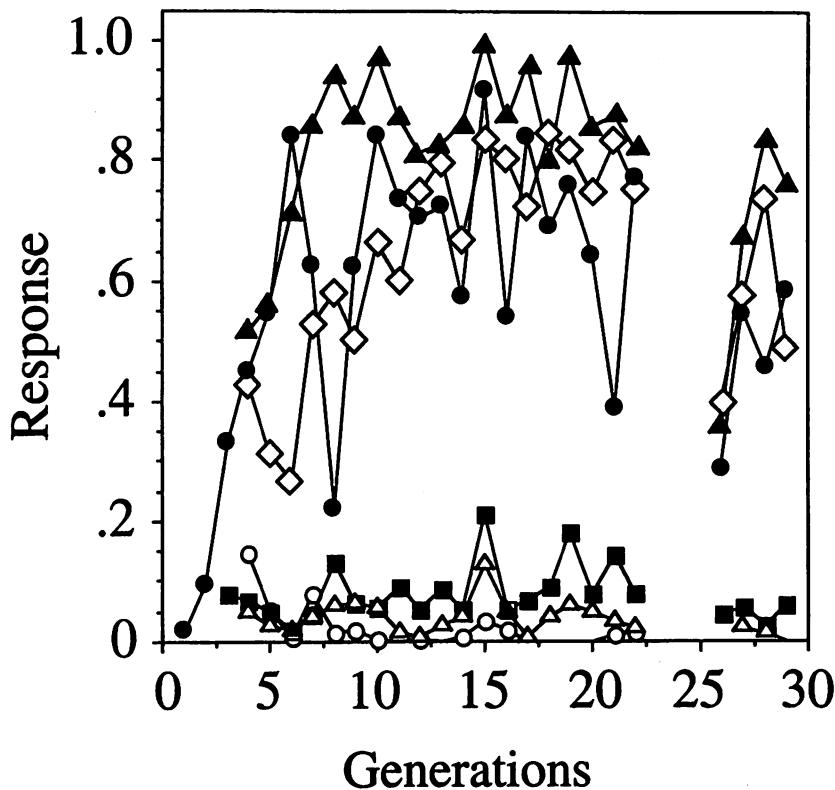


Figure 11.3 The incidence of Hsp90-induced eye deformities increases in selection experiments. Filled circles, open diamonds, and filled triangles indicate populations selected for high incidence of eye deformities. Open circles, filled squares, and open triangles indicate three control populations selected for low incidence of eye deformities. The horizontal axis shows time in generations, the vertical axis the fraction of flies with deformed eyes. The frequency of abnormalities in the low incidence lines does not approach zero, because all lines were initiated with a single affected male, and because alleles causing abnormalities can become fixed due to genetic drift in the small population used. From Figure 2 in (471).

geldanamycin and radicicol. This treatment generated multiple morphological abnormalities, including variation in the shape and color of cotyledons, the color and presence of true leaves, root morphology, and the orientation of roots and whole seedlings. Individuals within one inbred population tend to share the same morphological abnormalities, but individuals in different inbred populations show different abnormalities. This is exactly the pattern expected if the morphological abnormalities have a genetic basis, that is, if

they are caused by genetic differences that occur among populations, but that are absent within populations due to inbreeding.

The buffering that heat shock proteins provide against genetic variation is not restricted to eukaryotes. Although prokaryotes have nothing like the elaborately orchestrated developmental processes of eukaryotes, the integrity of their proteome also depends on proteins that assist other proteins in folding. One such protein in the bacterium *Escherichia coli* is groEL—a product of the groE operon—which is part of a large protein complex important for protein folding. Fares and collaborators (146) demonstrated that groEL can provide robustness against mutation. They took an approach different from the experiments I surveyed above, which demonstrated robustness in the wild-type by disrupting it in mutants, thus causing developmental abnormalities and reduced fitness. Instead, these authors reinstated robustness to mutations and fitness in mutant organisms. They used mutation accumulation populations of the bacterium *E. coli*. These are bacterial populations in which individual cells have accumulated—over many laboratory generations—multiple slightly deleterious mutations (281). As a result, these populations suffered a 50% reduction in fitness—as indicated by their slower cell division rate—relative to an ancestral population. Overexpressing GroEL in cells of these populations led to a restoration of their fitness to about 80% of the ancestral population. These experiments suggest that groEL can restore fitness losses that arose through mutations. Thus, heat-shock proteins may provide mutational robustness even in organisms without elaborate development and a macroscopic phenotype.

Taken together, all these experiments demonstrate that development and fitness of wild-type organisms is robust to cryptic genetic variation, genetic variation that becomes exposed by mutating suitably chosen proteins. Heat-shock proteins are a prominent class of such proteins. Mutations that reduce their activity can adversely affect many different phenotypic traits. Conversely, an increase in their activity through overexpression can reinstate an organism's high fitness, if mutations have previously reduced its fitness.

### The Many Genes Behind Monogenic Diseases

Hsp90 is an unusual protein because it renders many different characters robust to abundant genetic variation, and it does so in vastly different organisms. However, it is just an extreme example of a ubiquitous phenomenon, namely that the phenotypic effects of genes—here, genes that affect traits like wings, eyes, and bristles—depend on other genes. Vast numbers of genetic studies have unearthed this phenomenon. It is called epistasis. Without epistasis, there would be no robustness of a

genetic system against mutations, because every single gene would affect a system's performance, and it would do so independently of other genes (186, 579, 591). Most documented cases of epistasis, however, do not go far in answering the central questions of this book—with one exception. They all illustrate that a genetic system's robustness is under genetic control, that robustness does not just arise whenever genes interact to produce a phenotype, and that natural selection can thus modulate mutational robustness. Because of this principle's importance, I will briefly review one key class of examples much closer to human interest than the wings of flies. These examples regard the genetics of human disease. They show that efforts to understand robustness in genetic systems are not just academic exercises. Let these examples stand for the thousands of other documented cases of epistasis in the literature.

Most genetics textbooks contain long lists of genetic diseases referred to as monogenic, diseases such as cystic fibrosis, phenylketonuria, and thalassemia. The label "monogenic" suggests that a mutation in one gene causes the disease, and that it does so regardless of other genetic factors. However, increasingly detailed genetic analyses of such diseases show that this picture is vastly oversimplified. One and the same mutation may cause severe disease in one individual and have a lesser or even no effect in another individual. The reasons are becoming increasingly clear in some well-studied diseases that are reviewed in (534, 603). A notably well-studied example is  $\beta$ -thalassemia, a common genetic disorder of hemoglobin production.

Human hemoglobin is a tetrameric protein consisting of two different types of polypeptide subunits called globins. The nature of these subunits changes during human development. In the human fetus, most hemoglobin—also known as fetal hemoglobin (HbF)—consists of two alpha and two gamma globin chains ( $\alpha_2\gamma_2$ ). Around the time of birth, this fetal hemoglobin is replaced by adult hemoglobin (HbA), most of which has two beta subunits or two delta subunits instead of the fetal gamma subunits ( $\alpha_2\beta_2$  or  $\alpha_2\delta_2$ ). The human genome contains two copies (four alleles) of the  $\alpha$ -subunit-coding gene, but only one copy (two alleles) of the  $\beta$ -subunit-coding gene.

Mutations that affect hemoglobin are widespread: Some 7% of the world population are carriers for hemoglobin-related diseases (626). Among these diseases, the  $\beta$ -thalassemias present the biggest public health problem. They result from an imbalanced production of the  $\beta$ -hemoglobin subunits due to mutations in the  $\beta$ -subunit-coding gene. The result is an excess of  $\alpha$ -globin chains, which aggregate in red-blood-cell precursors, cause abnormal cell maturation, and lead to the premature destruction of these precursor cells. The  $\beta$ -thalassemias have extremely diverse clinical phenotypes. These range from profound anemia that, if untreated, is lethal

in the first year of life, through a spectrum of anemias with decreasing severity, to symptomless forms. The primary symptom of anemia comes with many other complications, including tissue damage from pathologically increased iron concentrations, bone disease, endocrine and cardiac damage, gall bladder disease, liver disease, and diabetes (603).

Part of the variation in  $\beta$ -thalassemia severity is caused by different mutations in the  $\beta$ -globin gene. More than 200 different such mutations are known. However, even individuals with the same  $\beta$ -thalassemia mutation can have widely varying clinical phenotypes. In fact, some individuals that produce no  $\beta$ -globin at all have only mild disease symptoms. Put differently, robustness to mutations that can cause severe disease varies widely among humans. Variation in genes other than the primary disease gene is partly responsible for such variation. Specifically, the mutations responsible for such robustness fall into two categories, genes that encode other hemoglobin subunits and genes unrelated to hemoglobin production.

An example of the first class of genes are those encoding the  $\alpha$ -globin subunit. In  $\alpha$ -thalassemia, the production of this subunit is reduced. A  $\beta$ -thalassemia genotype that also harbors an  $\alpha$ -thalassemia mutation often has less of an imbalance in  $\beta$ -globin production, thus alleviating the disease phenotype. Another example is mutations that compensate for reduced  $\beta$ -globin production through increased production of fetal hemoglobin. Some individuals with severely reduced  $\beta$ -globin production but mild disease continue to produce the fetal  $\gamma$ -globin and thus fetal hemoglobin. Some of the mutations alleviating the disease are associated with the  $\gamma$ -subunit encoding genes; others are not characterized, but map on chromosomal regions different from the globin coding genes (90, 91, 122).

Multiple mutations unrelated to globin production influence the severity of the disease and of its complications. One such complication is gall bladder disease caused by hyperbilirubinemia, an elevated level of bilirubin in the blood. Bilirubin is an intermediate in the breakdown of heme, a coenzyme necessary for hemoglobin function. The level of bilirubin in affected individuals is modified by mutations in the UGT1 gene, whose product is responsible for the glucuronidation of bilirubin, an essential step in the breakdown of bilirubin.

Another complex of complications, including cardiac disease and hepatic disease, is due to pathologically high iron concentrations. Mutations in the gene HFE, which is involved in hereditary hemochromatosis, a disorder characterized by excessive iron absorption from the diet, are responsible for variation in iron levels among affected individuals. A third complication is progressive osteoporosis in young adults. Polymorphisms at gene loci involved in bone formation and maintenance, including genes for the vitamin D receptor, collagen, and the estrogen receptor, influence its severity (603).

Taken together, these examples show how robustness of a healthy, multifaceted phenotype to genetic disease can be modulated by mutations in multiple, superficially unrelated genes. Thalassemia is just one of the best characterized among a growing list of "monogenic" diseases where one and the same mutation may show vastly different phenotypic effects. Another such disease is cystic fibrosis, the most common genetic disease affecting the Caucasian population of the United States. It is caused by mutations in the gene encoding the cystic fibrosis transmembrane regulator, a protein involved in the transport of chloride ions. The disease's key symptoms include progressive—and often lethal—deterioration of lung function, pancreatic insufficiency, and male sterility. The same disease genotype can be associated with widely differing degrees of lung function (278). The genes responsible for such variation are poorly characterized, but candidates are involved in susceptibility to respiratory tract infections. Such infections are partly responsible for lung deterioration.

Another classic "monogenic" disease is hyperphenylalaninemia, caused by defects in the enzyme phenylalanine hydroxylase (PAH), which converts phenylalanine into tyrosine. The main biochemical manifestations of this disease are increased serum levels of phenylalanine. Its most severe clinical manifestation is severe phenylketonuria (PKU), where the increased phenylalanine level results in brain damage and mental retardation. More than 60 mutations in the PAH gene are associated with PKU. In general, an individual's genotype is strongly associated with its biochemical phenotype, especially the phenylalanine level (412). However, at the same time, the genotype is a poor predictor of intellectual phenotype. For example, some individuals with less than 1% of normal PAH activity show only mild mental retardation. Moreover, within families with siblings of identical genotype and identical biochemical phenotype, individuals show very different intellectual development. The genes responsible for such variation are not characterized, but candidates are not difficult to come by. Absence of PAH activity causes reduced levels of tyrosine, a precursor for several neurotransmitters. The resulting reduced neurotransmitter production may be partly responsible for the ensuing mental retardation. Consequently, mutations that enhance the synthesis of alternative neurotransmitters or mutations activating alternative pathways of phenylalanine catabolism may modulate disease severity (534).

Further examples of complex monogenic diseases include Gaucher disease, a common lipid storage disorder, where merely one-third of homozygotes for the Gaucher mutation in Ashkenazi Jews are affected. The remaining two-thirds have mild, late onset, or no symptoms (45). Similarly, within families with an autosomal dominant mutation in the fibrillin gene that causes Marfan syndrome—a connective tissue disorder—some

members have severe ocular, cardiovascular, and skeletal symptoms, while others are only mildly affected (450).

All these examples illustrate how mutations that affect a genetic system's mutational robustness can have severe and often lethal consequences for humans. Together with the above examples from various animal systems—from Waddington's early experiments to more recent work on Hsp90—they illustrate that genes play key roles in determining robustness of an organism to mutation. The lessons from the animal systems, however, go beyond those from human disease. First, they show that one and the same gene can cause robustness of multiple phenotypic features to mutations. Second, the variation that is unveiled when this robustness is disrupted becomes a readily available substrate for natural selection.

# 12

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## The Many Ways of Building the Same Body

What is robust? An organism's body plan.

What is it robust to? Both minor and massive changes in embryonic development, which are ultimately caused by genetic changes.

This chapter deals with a level of biological organization far removed from DNA: many-celled organisms and their embryonic development. It makes two central observations. First, there are many and sometimes radically different ways to build the same body or body part. Nature has found some of these ways and transitions between them on surprisingly short timescales. This observation connects this chapter's examples to a common thread of previous chapters, a thread I revisit in chapter 13. Specifically, the observation provides a hint that the process of building a body shows great robustness to genetic changes. In other words, there must be many genetic changes that affect embryonic development but are neutral otherwise, in the sense that they do not affect the adult body plan. If this was not so, some of the changes in development I survey below could not have taken place.

The second observation is that such variation is a key to innovation. This is perhaps nowhere clearer than in the two last examples of this chapter. Although the body plan and morphology of an organism may remain unchanged, other features of the organism change radically as a by-product of changing the building process. If we will ever fully understand the mechanics of body plan formation, we may thus see principles similar to those governing lower levels of biological organization: Most biological problems have a vast number of different solutions, and many of these solutions harbor the seeds of innovation to solve other problems.

As we ascend from the molecular level to ever-higher level of organismal organization, our mechanistic understanding of the processes that produce organismal traits gets increasingly murky. The previous two chapters already point to this problem. More than a thousand man-years of research have not been sufficient to fully understand a network of a

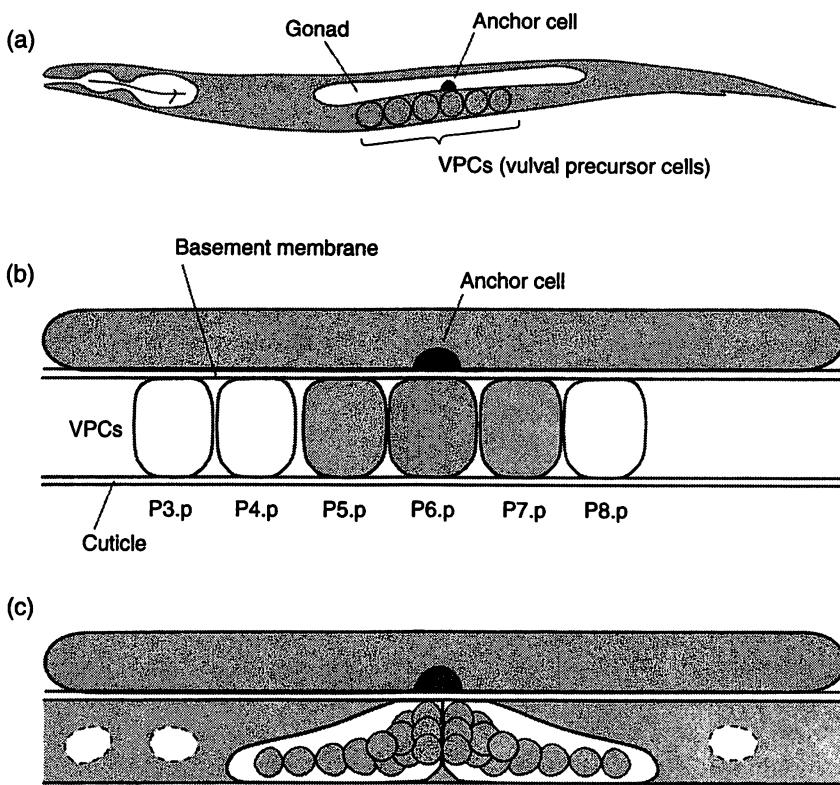
few dozen genes, such as the network that patterns the early *Drosophila* embryo. However, the development of even the simplest macroscopic feature, such as a hair, requires countless more genes. It is then hardly surprising that we poorly understand the genetic basis of most macroscopic features. All examples of this chapter regard such macroscopic features, and two of them regard the defining feature of many-celled organisms, their body plan. The examples also all lack the beauty of simplicity and complete knowledge. We do not know most of the genes involved in specifying body plans, how mutations in these genes affect the body plan, and whether robustness of a body plan is an evolved property. Despite such inadequacies of our knowledge, the phenomena I discuss below are too important to ignore in any discussion of robustness.

### Vulval Development in the Worm *Caenorhabditis elegans*

The first example regards not massive changes in developmental of an entire body plan, but changes in building an important body part, the vulva of the nematode worm *C. elegans* (303). The example illustrates how variation in development can occur among genetically identically organisms within a population and how such variation may permit developmental differences between species to evolve, differences that leave the final product—the vulva—unchanged. It thus illustrates on a small scale a process that takes place on a much larger scale in the other examples of this chapter.

*C. elegans* develops through a series of completely defined and invariant cell divisions. That is, any two worm embryos develop through the same stereotypical pattern of cell divisions, one of the peculiarities that makes this organism attractive to developmental biologists. One feature of the adult worm, its vulva, has received particular scrutiny. The vulva is part of the egg-laying apparatus, which consists of the uterus, the sex muscles, the vulva itself, and various neurons. The vulva itself forms from a small number of cells which form a tubular structure that connects the egg-producing gonad to the outside world. (Figure 12.1). Most individuals in a *C. elegans* populations are self-fertilizing hermaphrodites, where one individual produces both sperm and eggs. The vulva is not necessary for survival of such individuals, such that defects in its development are easily studied.

One strain of *C. elegans* whose development has been intensely studied is the genetically homogeneous N2 strain. In this strain, both the gonad and the vulva are derived from descendants of the same cell lineage. The vulva itself forms from six cells called vulval precursor cells, named P3.p to P8.p (Figure 12.1). One gonadal cell, the anchor cell, is important to vulva



**Figure 12.1** Vulval precursor cells and their descendants. (a) Location of the gonad, anchor cell, and vulval precursor cells in the second instar larva of a *C. elegans* hermaphrodite. (b, c) relationship of the anchor cell to the six vulval precursor cells (P3.p through P8.p). Dark shading of a precursor cell indicates its fate as a central vulval cell, light shading indicates lateral vulval cell fate, and white indicates hypodermal cell fate. The outline of the vulva is shown in the fourth instar larva. From Figure 17.33 of (189) and (271).

formation and lies directly above the vulval precursor cells. The cell directly underneath the anchor cell (P6.p) forms the central vulval cells; the two cells adjacent to it (P5.p and P7.p) will form lateral vulval cells. The three remaining cells (P3.p, P4.p, and P8.p) will fuse with other cells and become part of a syncytial hypodermis (the *C. elegans* epidermis). Signals from the anchor cell and communication between the vulval precursor cells themselves are necessary for vulva formation. For example, if the anchor cell is killed, all vulval precursor cells form hypodermal cells. Importantly, the pattern of vulval formation shown in Figure 12.1 is not rigid. Elimination of

the three vulval precursor cells P5-7.p, for example, will change the fate of P3.p, P4.p, and P8.p, such that they will become central and lateral vulval cells. In general, any one of the vulval precursor cells is capable of adopting any of the three possible cell fates. In this sense, the vulval precursor cells form a group of cells with the same developmental potential.

The anchor cell itself develops in a curious fashion (611). It can be derived from either of two gonadal precursor cells called Z1.ppp and Z4.aaa. One of these cells eventually expresses a receptor molecule, LIN-12, and the other its ligand, LAG-2. The LAG-2-expressing cell becomes the anchor cell, whereas the LIN-12-expressing cell becomes a precursor of uterine tissue. However, shortly after their formation—before their ultimate fate is determined—Z1.ppp and Z1.aaa are completely equivalent in the sense that they express both LAG-2 and LIN-12 in equal amounts. So how do they come to adopt different fates? The answer is that small and random differences in receptor and ligand expression level between the two cells are amplified by feedback loops, such that each cell eventually expresses only the ligand or receptor. Whether Z1.ppp or Z4.aaa becomes the anchor cell is thus completely random. In 50% of genetically identical individuals, Z1.ppp will become the anchor cell, and in the other 50% Z1.aaa will.

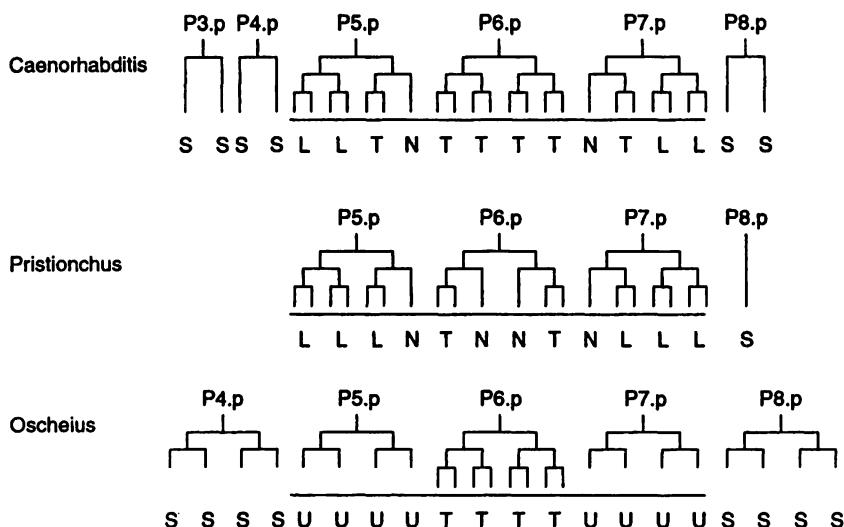
Differences in vulval development have been studied within populations and strains of *C. elegans*, as well as in multiple species related to *C. elegans*, some of which I discuss below. The most prominent among these species are *Oscheius sp.* CEW1, which belongs in the same family as *C. elegans* (Rhabditidae), as well as *Pristionchus pacificus* (Diplogastridae). Despite a long history of phylogenetic studies of the phylum nematoda (50, 530, 531), its phylogeny is still in flux, and absolute divergence dates are currently not available for these species.

Some differences in vulva development occur at low frequencies (0.1–1%) within populations (108). For example, in *Oscheius sp.* 1 central vulval cells are sometimes not formed from the P6.p precursor, but instead from P5.p or P7.p, which form lateral vulval cells in *C. elegans*. Also, one of the vulval precursor cells may be missing in some individuals and is then replaced by another cell (108). The resulting adult vulva is normal. Other developmental differences occur at much larger frequencies. For example, in 50% of *C. elegans* individuals, the cell P3.p divides once and is competent to form vulva cells (although it will normally become part of the syncytial epidermis). In the remaining 50%, P3.p joins the epidermal syncytium without dividing and is not competent to adopt a vulval cell fate (532). The best-known example of abundant developmental variation regards the origin of the anchor cell: As I discussed above, in 50% of *C. elegans* individuals, Z1.ppp will become the anchor cell, and in the other 50% Z1.aaa will.

Most such examples of intrapopulation variation reflect not genetic variation but random noise in development. In cases like anchor cell formation, the source of this noise is well understood. It reflects random differences in gene expression levels across cells that become subsequently amplified. Such noise demonstrates that developmental processes such as vulva and anchor cell formation have built in flexibility. This flexibility may lead to the evolution of developmental differences between strains and species. For example, whereas in the *C. elegans* reference strain N2 50% of P3.p cells divide before melting into the hypodermis, in strain CB4857 only 15% of cells do (108). In *Caenorhabditis briggsae* 0–15% of P3.p cells divide, whereas in *Caenorhabditis sp.* 3 all P3.p cells divide. Regardless of P3.p division frequency, however, *C. briggsae* P3.p cells are never competent to adopt a vulval fate, whereas they have this competency in *C. elegans* (108). Similarly, whereas in *C. elegans*, Z1.ppp and Z1.aaa are equally likely to become anchor cells, in species of the closely related genus *Acrobeloides* Z4.aaa becomes the anchor cell in most animals, and in *Cephalobus cubensis* Z4.aaa always becomes the anchor cell (147, 149).

An analogous pattern of variation holds for division of the P4.p and P8.p cells in the genus *Oscheius*. Within some species, intra- and inter-strain variation exists in the number of divisions these cells undergo. For instance, in some individuals they undergo no, in other individuals they undergo one, and in yet others they undergo two cell divisions before becoming part of the hypodermis. In other species, such polymorphisms have become fixed. For example, in *O. guentheri* SB1333, P8.p cells do not divide at all, whereas in *O. sp* PS1017 they undergo one cell division, and in *O. myriophila* DF5020 they undergo two cell divisions (107, 108). Variation in cell division patterns can also be elicited by treatments with mutagens like ethyl methane sulfonate (116).

Figure 12.2, from a recent literature review (512), summarizes changes that occur in cell division patterns in the three species where vulval precursor cell fates are best understood, *C. elegans*, *Oscheius sp.* CEW1, and *P. pacificus*. Such changed cell division patterns, however, reflect only some of the modifications vulval development has undergone. Others involve the cell interactions that are necessary for determining vulval precursor cell fates. Vulval cell fate determination requires the anchor cell in *C. elegans*. In contrast, some strains of *Panagrellus redivivus* do not require the anchor cell for vulval patterning (148). In *Pristionchus pacificus*, vulva formation requires continuous interaction of vulval precursors with the gonad, an interaction that starts long before the anchor cell is born (500). Conversely, in some species of the genus *Panagrolaimus* (148), the gonad is altogether dispensable for formation of lateral vulval cells (107).



**Figure 12.2** Comparison of vulval cell lineages between *Caenorhabditis elegans*, *Pristionchus pacificus*, and *Oscheius* sp. CEW1. L, longitudinal division; N, no division of the Pn.p granddaughters; S, cells fusing with the hypodermal syncytium; T, transverse division; U, no division, but cells lack the typical characteristics of N cells as described in *C. elegans*. Cell lineages absent in *P. pacificus* indicate that the respective cells undergo programmed cell death. From Figure 3b in (512).

In sum, there is much variation within species in cell divisions and cell interactions that lead to vulva formation. Some of this variation translates into between-species differences in vulva development. Despite such sometimes substantial differences, the end result of development is a vulva with essentially the same structure and function. In this sense, one can view all these developmental differences between species—which are ultimately caused by genetic change—as neutral with respect to the adult feature, the vulva. These examples from vulval development scratched only the surface of the burgeoning field of comparative developmental studies in nematodes (107, 147, 510, 511). Many of the molecular interactions involving vulva formation are being intensely studied (303), which will help in elucidating the molecular mechanisms behind this change.

As an example of developmental reorganizations, vulva formation is not as dramatic as the next two examples. If one compares the building of an organism to the building of a house, then variation in vulva formation shows how bricks can be locally rearranged to build the same structure. The next examples show how the entire house can be built in completely different ways. Because evolution has readily found many

ways to build the same organ and organism, the underlying developmental process must be robust to at least some of the random changes that affect it. Fixed differences among species and neutral variation within species hint at such robustness.

## Parasitic Insects

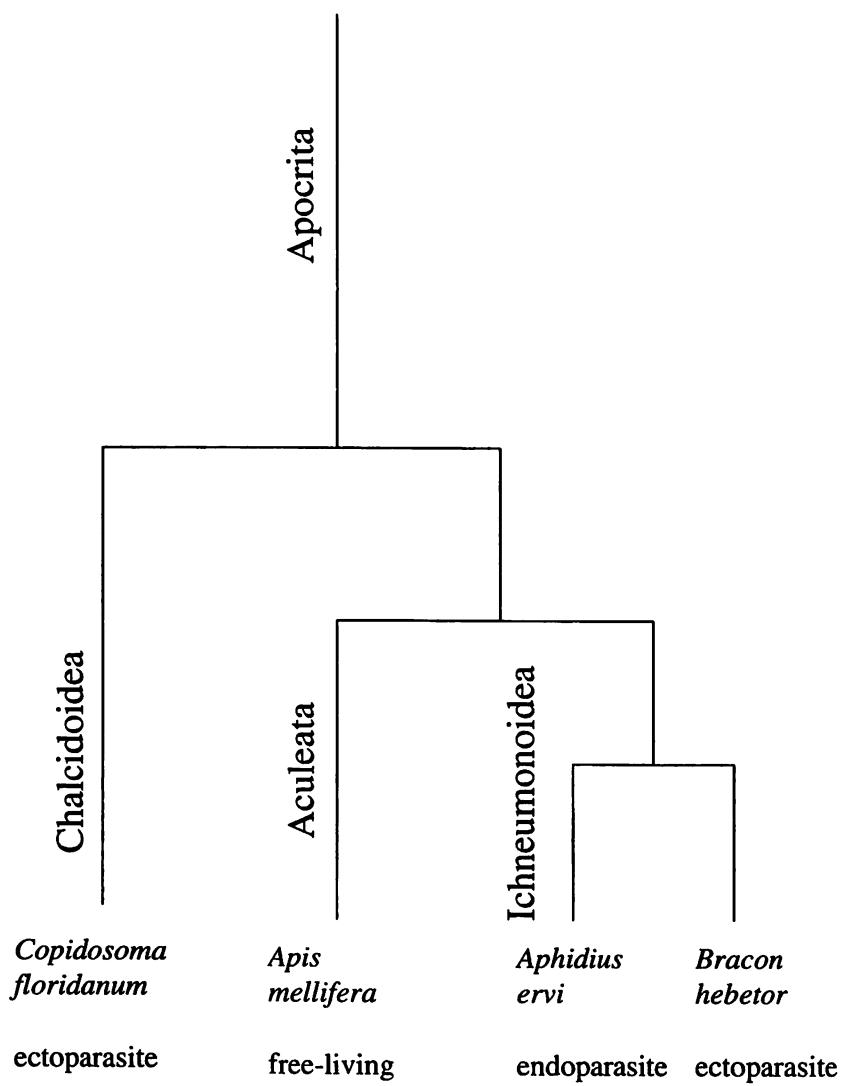
The stars of this section belong to the insect order hymenoptera (ants, bees, and wasps), which originated some 220–300 million years ago (605). More precisely, they belong to the suborder Apocrita (Figure 12.3). This suborder comprises free-living insects such as bees, but also a diverse group of wasps that parasitize other insects. Such parasitic wasps fall into two major groups: ectoparasites that lay eggs on their host's surface, and endoparasites that deposit their eggs into the host.

The early development of some hymenoptera, such as honeybees, and some ectoparasitic wasps, such as *Bracon* (Figure 12.3), shares many features with that of fruit flies, some of whose aspects I briefly reviewed in Chapter 10. First, like flies, these hymenoptera have a large egg, with a large yolk mass in the egg's center. A hard, protective envelope, the chorion, protects the egg from the exterior environment. Second, the unfertilized egg already has polarity: It possesses a distinct anterior and posterior end. (In the case of *Drosophila*, this polarity comes from gene products deposited by maternal cells on either end of the egg.) Third, as the fertilized egg begins to divide, the first nuclear divisions cannot traverse the egg completely, because of the large yolk mass: In embryological terms, cleavage in such insects is superficial (189).

Fourth, the earliest cell divisions take place in a syncytial embryo, where cell walls have not yet form between newly divided nuclei (158). This has important consequences for early patterning mechanism, because it implies that signaling across cell membranes is not necessary to pattern the embryo. Instead, much of the communication between different embryonic regions takes place through free diffusion of regulatory molecules.

Fifth, both the fly and the honeybee belong to a subgroup of insects called long-germband insects (478). In these insects, the primordia of all later segments are already present after the first cell divisions. Put differently, segmentation occurs by subdividing the initially homogeneous embryo—through a well-understood mechanism in the case of *Drosophila*. In contrast, in other insects—intermediate and short-germband insects such as the grasshopper (order Orthoptera)—posterior segments grow out of an embryo's anterior part.

Sixth, long-germband insects share expression features of key genes necessary for segmental patterning. For example, despite some differences



**Figure 12.3** Phylogenetic relationships among the hymenopteran taxa discussed in the text. They belong to the suborder apocrita, which comprises, among others, the superfamilies Aculeata (including free-living bees and wasps), Ichneumonoidea, and Chalcidoidea. Parasitic families within the latter superfamilies include both ectoparasitic species (*Bracon hebetor*) with large and yolk eggs, and endoparasitic species (*Copidosoma floridanum*, *Aphidius ervi*) with small and yolk-poor eggs. From information in (208, 605).

in its expression, the honeybee *even-skipped* gene is expressed in a two-segment-wide striped pattern similar to that of *Drosophila*. Its expression later in development in neurons and dorsal cells also mirrors that in other long-germband insects like *Drosophila* (47, 208). Such similarities suggest that embryonic patterning events mediated by *even-skipped* are similar in fruit flies and honeybees.

The development of some parasitic wasps deviates sharply from this canonical pattern of *Drosophila*, and even from the development of other closely related parasitic wasps such as *Bracon* (208). A case in point is the endoparasitic wasp *Copidosoma floridanum*. Its adults are 2-mm-long wasps that deposit a single egg into the egg of a moth host. As the host egg develops into a larvae, so does the parasite inside it, consuming the host's tissues until the parasitic larvae pupate. Eventually, the adult wasps emerge through the cuticle of the host's cadaver. Despite the overall similarities in larval and adult body plan to many other insects, the wasp's body plan forms in drastically different ways. The first indication of these differences can already be seen in the egg. While the eggs of fruit flies, honeybees, and ectoparasitic hymenoptera such as *Bracon* are large and yolk, that of *Copidosoma* is tiny and poor in yolk. Unlike that of other insects, it is also not protected by a thick chorion. Second, as a consequence of the small amounts of yolk, the first cell divisions are not superficial but total: They completely traverse the egg. Moreover, early development does not proceed in a syncytium (209). Nuclei are separated by cell walls as early as the four-cell stage. This must have effects on how the future segments are patterned, because simple diffusion of transcriptional regulators throughout the embryo is now impossible.

All these changes, however profound, pale in comparison to the truly bizarre variation on development characterizing this species: polyembryony. The one egg inside the host does not become one larvae, but its cells instead divide rapidly into some 2000 distinct cell aggregates. Each of these aggregates then forms one embryo, which develops into a larva. Put differently, some 2000 larvae can emerge from the one egg deposited into a moth host. Not only does this mean that the simple embryonic subdivision characterizing long-germband insects cannot possibly explain embryonic patterning, it also means that the initial egg polarity, if established in a fashion similar to that of *Drosophila*, is irrelevant for later embryogenesis. In fact, the axes of the 2000 embryos are randomly oriented relative to the original egg's polarity (209, 210).

Given such drastic changes, it comes as no surprise that expression patterns of developmental genes also show deviations from those seen in other insects. For example, *even-skipped* in *Copidosoma* is expressed in a one-segment-wide pattern resembling that of segment polarity genes

(208, 209), but not in a two-segment-wide striped pattern as in the honeybee and *Drosophila* (chapters 7 and 10).

Even more drastic changes in gene expression occur in other endoparasitic wasps. A case in point is *Aphidius ervi*, an endoparasitic wasp closely related to the ectoparasitic *Bracon hebetor*, which develops similarly to *Drosophila* and fruit flies (211). *Aphidius*, like *Copidosoma*, develops very differently from *Drosophila*, as indicated by its small yolk-poor eggs, total cleavage, and early cellularization. Expression of *even skipped* in *Aphidius* never shows a striped pattern at all (211).

Many insects do not share the canonical developmental patterning of the well-studied *Drosophila*. This insight from classical embryological studies (478) is further underscored by molecular studies. For example, key *Drosophila* segmentation genes such as the pair rule gene *fushi tarazu* either have adopted completely different expression patterns or have evolved almost beyond recognition in members of other insect orders, such as the short-germband grasshopper (100). However, the different insect orders are old, having emerged several hundred million years ago (605). That they might have developed different patterns of development is thus less surprising than for the closely related species I discuss here. For example, the ectoparasitic *Bracon*, which shares many features of *Drosophila* development, and the endoparasitic *Aphidius*, whose development differs radically, come from closely related sister genera within the same family (Braconidae). Unfortunately, absolute divergence dates for these taxa are not available.

## Direct Development in Sea Urchins

Sea urchins comprise about 1000 species, among which radical reorganizations of development have frequently occurred (453, 627). This is not to say that some aspects of development have not been conserved. In fact, some such aspects have been conserved for over 250 million years of sea urchin evolution. They include radial cleavage—the first cell divisions after fertilization—a cleavage pattern typical for a deuterostome. In radial cleavage, the axes of the first cell divisions are perpendicular to each other. Another such feature is an unequal fourth cleavage, which leads to a 16-cell embryo whose cells are of unequal size (Figure 12.4). More specifically, the 16-cell embryo is subdivided into a vegetal half that consists of four macromeres and four micromeres, and an animal half with eight mesomeres (189). The fates of these 16 cells are different. Mesomeres, for example, will form the larval ectoderm, macromeres will give rise to the gut and part of the coelom, and micromeres will give rise to the skeleton. After several more rounds of cell division, the embryo

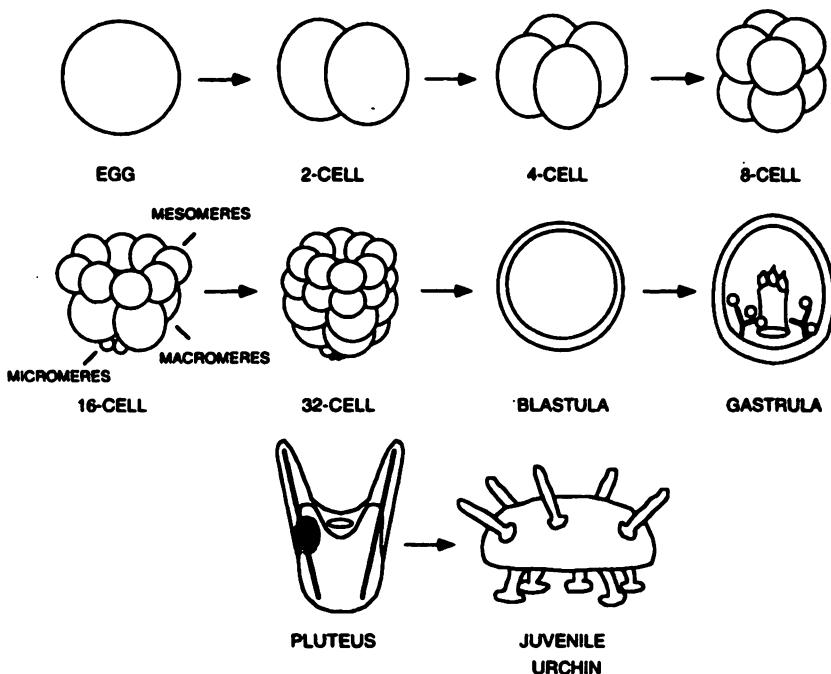
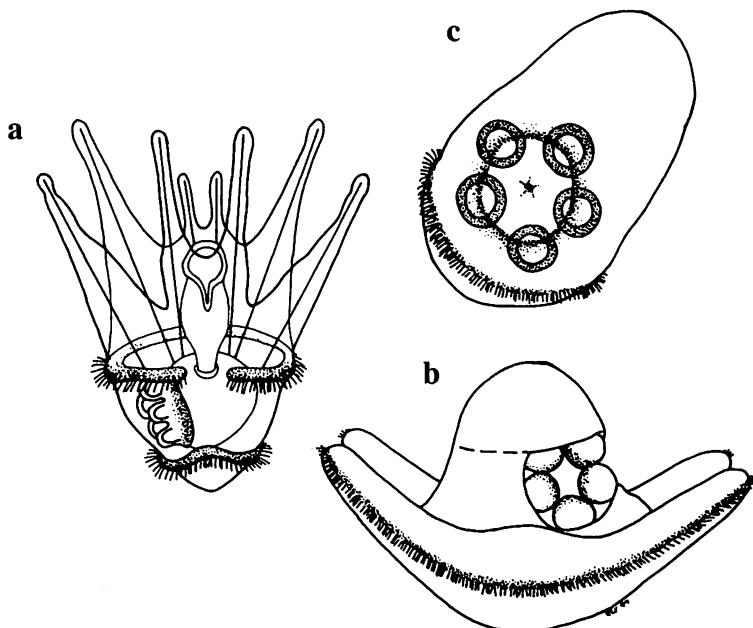


Figure 12.4 Indirect development of a typical sea urchin. From Figure 7.2. of (453). Copyright 1996 by the University of Chicago.

forms a hollow blastula, which invaginates during gastrulation and eventually forms a plankton-feeding, free-swimming larva, the pluteus, another characteristic of this organism. The pluteus possesses eight arms that are supported by an internal skeleton, as well as a band of cilia for swimming and food capture (Figure 12.5). Inside the larva, a structure called the echinus rudiment forms from part of the coelom and from the vestibule, an ectodermal invagination. This echinus rudiment will later become the adult sea urchin (453).

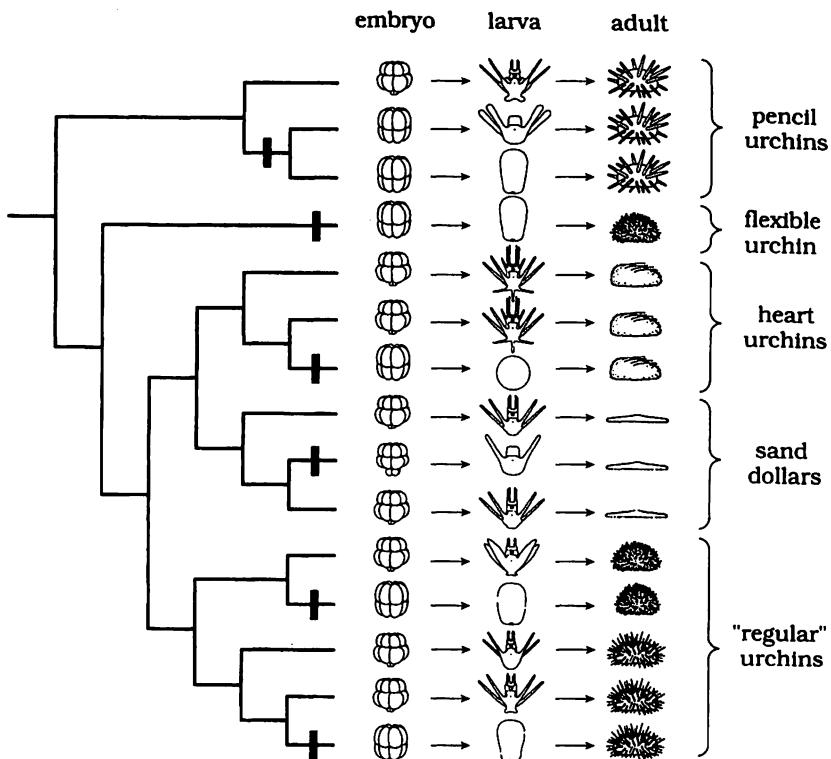
This prototypical mode of development has been massively altered several times and independently in multiple lineages (Figure 12.6). In some of these lineages, nonfeeding larvae have evolved that share key features with the pluteus, such as several arms, an internal skeleton, and a band of ciliae. Some sea urchin species, however, have completely abandoned indirect development through a pluteus larva and develop directly from the egg (Figure 12.5c). Some of the species with different developmental modes are very closely related. A case in point are two direct-developing Australian pencil urchins within the genus *Phyllacanthus* (414, 433). One of them has a nonfeeding pluteus larva with four arms and a band



**Figure 12.5** Three kinds of larvae in sea urchins. (a) A typical feeding pluteus of *Strongylocentrotus droebachiensis*. The rudiment that will give rise to the juvenile sea urchin and its five primary podia are visible through the largely transparent body, adjacent to the stomach. (b) The nonfeeding partial pluteus of *Phyllacanthus imperialis*, with the juvenile rudiment lying in the oral region between the reduced pluteus arms. Several pluteus features are retained, including four arms, an internal larval skeleton, and a ciliary band running along the arms. (c) The direct developing larva of *Heliocidaris erythrogramma*. A few pluteus features are retained: rudimentary larval skeletal elements, a larval nervous system arrayed along a relict larval oral-aboral axis, and a reduced ciliary band. Figure and legend from Figure 7.4. of (453). Copyright 1996 by The University of Chicago.

of ciliae (Figure 12.5b), whereas the other has a football-shaped larvae without any pluteus-like feature.

Perhaps the best-studied closely related pair of species with different developmental modes belong to the genus *Heliocidaris* (227, 453, 629, 630). They inhabit shallow waters in southeastern Australia. One of them, *H. tuberculata*, produces a typical feeding pluteus. The other, *H. erythrogramma*, develops in a few days directly from the egg without a pluteus-like larva. These two species are striking because they are very closely related. Similarities between their mitochondrial and nuclear DNA (367, 503) suggest that they shared a common ancestor as little as 5 million years ago. (Five million years is of the same order of magnitude



**Figure 12.6** Evolution of developmental mode in sea urchins. Adult and larval forms are mapped on a phylogeny containing the major living sea urchin orders. Adult morphology is conserved in most lineages, but sand dollars and heart urchins have greatly modified adults. The pluteus larva is maintained by most lineages, and is the primitive mode of development. Black bars indicate lineages with direct development, which has arisen independently more than 20 times. Figure courtesy of Greg Wray. From Figure 7.3 of (453). © 1996 by The University of Chicago.

as the divergence between humans and chimpanzees—just imagine chimpanzees developing indirectly through a feeding larva utterly alien in appearance to the adult.) Despite their recent divergence, the developmental changes between these two species are profound.

The earliest change regards egg size. The directly developing *H. erythrogramma* has eggs a hundred times larger than the indirect developer *H. tuberculata*. Such an increase in egg size is typical among direct developers. They cannot acquire nutrients needed for development through feeding, and thus need to store them in the egg. A second change regards

the overall course of development. *H. erythrogramma* has completely abandoned some features characteristic of sea urchin development, such as unequal cleavage. That is, all cells in its 16-cell embryo are equally large. Such size changes, however, are just a reflection of more profound developmental changes, changes that cannot be fully explained through a simple loss of larval features or through heterochrony. (Heterochrony is an altered timing of development that is often held responsible for profound morphological changes in the adult [189].) Instead, the entire developmental program has been rearranged in *H. erythrogramma* (630). This is nowhere clearer than in a map of cell fates in the 32-cell embryo for *H. erythrogramma* and *Strongylocentrotus purpuratus*, a well-studied prototypical indirect developer (Figure 12.7). For example, the ciliary band in the indirect developer *Strongylocentrotus purpuratus* develops mostly from animal pole cells, cells formed at the yolk-poor part of the egg (upper part of the embryo in Figure 12.7), whereas in the direct developer, it forms from vegetal pole cells. The larval mouth has been completely lost in the direct developer. In the indirect developer, 16 of 32 cells will become ectoderm, whereas a full 26 of 32 do so in the direct developer. In the indirect developer, only part of two cells form the vestibule, from part of which the adult forms, whereas fully 8 cells do so in the direct developer. The fates of cells that will later form the nervous system, the skeleton, and the coelom have also been substantially rearranged, as can be seen in Figure 12.7. For example, the micromeres, the smallest cells in the 16-cell stage of indirect developer, give rise to coelomic cells and skeleton-secreting cells. In the indirect developer, no one cell type exists that corresponds to the micromeres and that gives rise to only these two types of cells. These are only some of many developmental changes from indirect to direct development. Others, including a changed blastula and gastrula formation, a late onset of skeletal formation, and changes in expression of developmental genes, are summarized in (453).

In sum, closely related species of sea urchins have evolved direct development, not through simple loss of larval features or heterochrony but through a complete reorganization of development that is already visible in its earliest stages. Despite these massive changes in development, an adult with essentially the same body plan emerges. As an aside, the ease of such rapid developmental reorganization may also shed light on the origination of body plans. For it is, of course, difficult to imagine how nature would have stumbled upon any one body plan, with its unfathomable complexity in the first place. The rapid invention of several dozen body plans during the Cambrian radiation, however, suggests that inventing new body plans may be easier than we think. Facile and radical reorganizations of development that continue to this day also point to

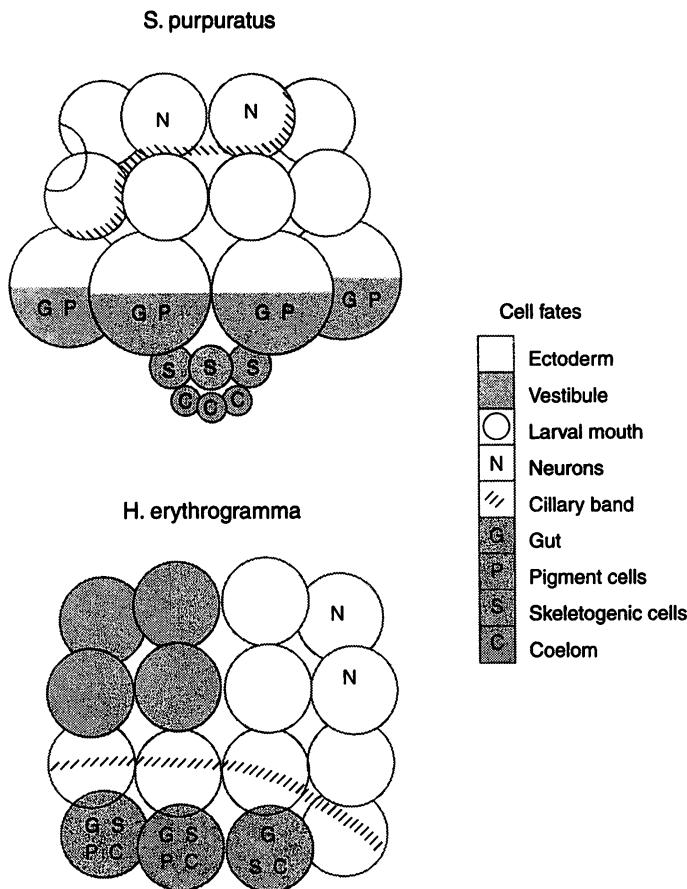


Figure 12.7 The larval cell fate map of the 32-cell *H. erythrogramma* embryo compared with that of an indirect-developing sea urchin 32-cell embryo. Larval cell types are indicated in the key. Figure and legend from Figure 7.6 of (453) and from (630). © 1996 by The University of Chicago.

this possibility. A better understanding of how such reorganizations occur may also help in understanding the origin of body plans.

### Reorganization of Development and Ecology

The above examples—chosen from closely related species whose development is well studied—are only the tip of the iceberg. Early development can differ profoundly among related species, and key developmental

differences, such as those between feeding and nonfeeding larvae, parasitic and free-living lifestyles, mono- and polyembryony, egg-laying and livebearing, and free-spawning and internal fertilization, have evolved multiple times in different animal phyla. Such differences in development may represent different and equivalent solutions to one problem—how to build a body. However, they differ in other, important ways. That is, such massive developmental changes are not just historical accidents, nor do they reflect a genotype's drifting through a vast space of developmental modes, but they are associated with fundamental changes in lifestyle. Take the case of endoparasitic wasps (208). As opposed to free-living and ectoparasitic insects, endoparasites deposit their eggs not into a harsh exterior environment, but in the nutritious tissue of other insects. It is thus easy to see why endoparasites can dispense with features such as the tough chorion and the nutrient supply of a yolk mass. Such changes facilitate other developmental modifications, such as total (not superficial) first cell divisions. Additional reorganizations, such as polyembryony, may ensure maximal exploitation of the host resource, as well as a competitive advantage in case the host has been infected by other parasites. Thus, while massive developmental reorganizations may do nothing to the adult body plan—and may be neutral in this respect—they are a consequence of adaptation to the endoparasitic life style.

A similar principle applies to the evolution of direct development in sea urchins. Direct development and indirect development through a larvae that swims and feeds in the ocean, represent fundamentally different life history strategies (453, chapter 7). Indirectly developing marine invertebrates are subject to high mortality of their larvae from predators. They thus need to produce large numbers of small eggs. Also, because their larvae spend much time in the water column, they can disperse widely, which is part of the reason why indirect developers have very broad geographic distributions and low species extinction probabilities. Direct developers, in contrast, have a much higher survival rate per embryo and produce a small number of large eggs that do not disperse as widely. In addition, direct developers are more abundant in the deep sea and in polar latitudes, perhaps because of lower plankton productivity there.

In sum, these rules make it clear that developmental change can have profound consequences on a species' life history. A neutral change with respect to one organismal feature—the adult body plan—can radically alter other aspect of an organism's biology. Such change can open completely new ways of life and new ecological niches. The developmental changes observed in sea urchins and parasitic insects are probably not caused by natural selection on the mode of development itself, but are a by-product of natural selection on other life-history aspects. However, such developmental changes are possible only if there are radically different

ways of building the same adult body, a vast neutral space of organizing development. This harks back to the central theme. For, if body plan formation was not robust to many genetic changes, how could radical reorganizations of development occur on very short evolutionary timescales and leave the adult body plan intact?

Our incomplete understanding of the genetic changes underlying developmental changes does, of course, leave a sense of dissatisfaction with all the examples I discussed. One can only hope that systematic genetic perturbations of developmental processes will eventually become possible on a large scale. Such perturbations would provide the ultimate proof that body plans and their development are no exception to the mutational robustness pervading lower levels of biological organization.

On a final note, I am well aware that my usage of the word neutrality throughout this and previous chapters may be objectionable. It is certainly different from a usage common in studies of molecular evolution, where a neutral change is a genotypic change without any effect on an organism's fitness. There are good reasons to abandon this common usage, as I will argue in more detail in chapter 14.



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## **Part III**

### **COMMON PRINCIPLES**



# 13

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## Neutral Spaces

I argue here that the following concept of a neutral space can provide a unified view of robustness in many different systems I discussed earlier:

A neutral space is a collection of equivalent solutions to the same biological problem. It can also be thought of as a set of alternative configurations of a biological system, configurations that solve the same problem.

Biological systems embody solutions to a wide variety of problems, from the faithful replication of genetic information to the making of a body. Most of these problems have more than one solution, and many of them have an astronomical number of equivalent solutions. Together, all of these solutions—equivalent with respect to a particular problem, but not necessarily in other respects—constitute a neutral space. Below I revisit, in ascending order through the hierarchy of biological organization, the neutral spaces associated with the examples of earlier chapters. After this tour through the previous chapters I discuss some limitations of the neutral space concept. Finally, I summarize how robustness of biological systems—pervasive on all levels of organization—is rooted in the structure of neutral spaces.

Mathematicians distinguish different kinds of spaces (391). All the spaces I discuss here are metric spaces. Loosely speaking, metric spaces are collections or sets of objects where any two objects have a well-defined distance. Metric spaces include our everyday three-dimensional space and higher-dimensional generalizations of it. However, they are not limited to such *continuous* spaces, where coordinates on every axis can assume an infinite number of continuous real values. In fact, many other metric spaces have properties very similar to the space of protein sequences first made popular by Maynard-Smith (363). First, they are *discrete*. That is, unlike our three-dimensional continuous space, each “coordinate” can assume only a finite and small number of values. Second, these metric spaces often have many—up to thousands—of dimensions. These two features, taken together, make for some of the counterintuitive properties we first encountered in the chapter on RNA structure. We will here encounter both continuous and discrete neutral spaces, most of which have many dimensions.

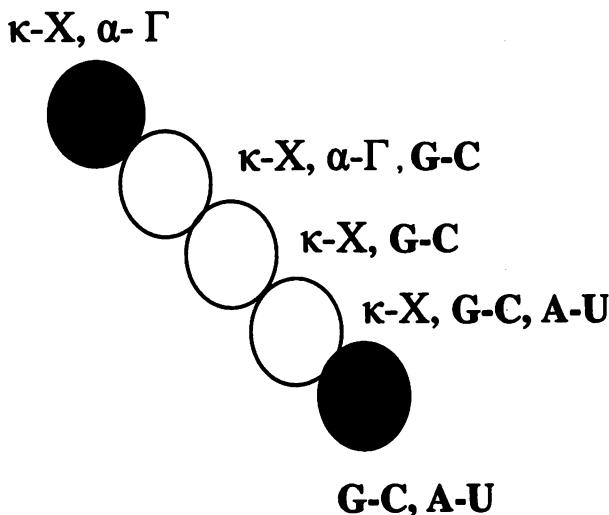
The first two examples in my tour through the previous chapters, genetic alphabets and genetic codes, are unusual for two reasons. First, with some exceptions, both the genetic code and genetic alphabets ceased to evolve a long time ago. In contrast, most other systems, from molecules to genetic networks continue to evolve to this day. In addition, genetic alphabets and genetic codes are not concrete objects like proteins. Rather, they are ways of representing and transmitting genetic information.

### Alternative DNA and RNA Alphabets

Recall from chapter 2 that the problem of faithful DNA replication may be solvable by alternative DNA alphabets. The four bases—A,C,G,U or T—that carry all genetic information are not the only possible bases that can be incorporated into genetic material. For example, polymerases can faithfully incorporate other bases into DNA and RNA, such as the base analogues  $\kappa$  and X.

A genetic alphabet that ensures faithful replication (robustness) through the principle of Watson-Crick base complementarity must fulfill two conditions (351): First, binding between complementary bases must be strong. Second, noncomplementary bases must repel each other. Thermodynamic considerations I discussed in chapter 2 suggest that Watson-Crick base pairing allows an alternative solution to this problem, a solution that fulfills these two conditions as well as the (G-A, C-U) alphabet we see all around us. This alternative solution is the chemical alphabet ( $\kappa$ -X,  $\alpha$ - $\Gamma$ ). It is not realized in life as we know it, perhaps because prebiotic conditions were not conducive to the synthesis of its bases, perhaps because of other chemical instabilities, or perhaps because of a mere historical accident. There are many other possible genetic alphabets, but all of them confer submaximal replication fidelity, for various reasons I discussed earlier (351).

We can represent an RNA alphabet as a point in a high-dimensional discrete space as follows. Order all stable bases from chapter 2 in some arbitrary way, e.g., alphabetically, (A,  $\alpha$ ,  $\beta$ , C, G,  $\Gamma$ ,  $\kappa$ , U, X). If an alphabet contains a particular base, write a one in the corresponding array, if not then write a zero. Thus, the (G-A, C-U) alphabet becomes (1, 0, 0, 1, 1, 0, 0, 1, 0), and the alternative ( $\kappa$ -X,  $\alpha$ - $\Gamma$ ) alphabet becomes (0, 1, 0, 1, 0, 0, 1, 0, 1). These two alphabets are two points in a 9-dimensional discrete space of  $2^9$  RNA alphabets. They form a small subspace, the neutral space of alternative optimal solutions for the problem of faithful genetic replication. Optimality is synonymous here with maximal robustness in



**Figure 13.1** A schematic illustration of the (small) neutral space of hypothetical alternative optimal solutions to the problem of faithful replication of genetic material. The filled circles represent the neutral space of optimal alphabets (G-A, C-U) and ( $\kappa$ -X,  $\alpha$ - $\Gamma$ ). The open circles represent a chain of alternative (suboptimal) alphabets that connect the two optimal alphabets and that differ from each other by the addition and deletion of pairs of complementary bases.

the case of DNA replication, in contrast to some of the following examples, where some optimal solutions to a problem may be more robust to mutations than others.

Note that the two alternative alphabets are not connected in the space of all possible alphabets. In other words, one cannot change from one to the other alphabet by adding or eliminating single bases or complementary pairs of bases. Figure 13.1 shows that at least four independent additions or eliminations of complementary pairs of bases are necessary to obtain one alphabet from the other. All alphabets that are intermediates of this transition between two alphabets are suboptimal. The alphabet of our genetic material evolved so long ago, and has not changed since, that we do not know whether nature explored different alphabets of DNA; nor do we know how transitions between alphabets might have occurred—by addition or elimination of individual bases, base pairs, or multiple bases at once. However, this disconnectedness of the neutral space of DNA replication suggests that transitions between the alphabets are difficult. Once evolution stumbled upon any one alphabet, the alphabet might have been here to stay.

## The Genetic Code

A genetic code is a map from all possible nucleotide triplets to 20 possible amino acids and a translation termination signal (Ter). A straightforward spatial representation of the nearly universal code realized in extant life could look as follows. Arrange all 64 nucleotide triplets in some order, such as the ascending alphabetical order, (AAA, AAC, . . . , UUU). Let each possible genetic code correspond to a point in a 64-dimensional discrete space, each of whose axes or coordinates corresponds to one of these triplets. The  $n$ th coordinate of this point contains the amino acid (or termination signal) encoded by the  $n$ th codon in the above order. In other words, each axis of this space admits 21 possible values (20 amino acids and one termination signal), corresponding to what the respective codon stands for. With the above codon order, the universal code corresponds to a point in this space whose first eight coordinates are (Lys, Asn, Lys, Asn, Thr, Thr, Thr, Thr, . . . ). This is because AAA encodes lysine (Lys), AAC encodes Asparagine (Asn), and so on.

A genetic code in modern day organisms with their 20 amino acids has to provide at least one codon for each of the twenty amino acids and at least one termination signal for translation. All codes—points in this 64-dimensional space—that have this property are valid codes, equivalent solutions to this problem. The neutral space of equally functional codes they constitute is a subspace of the space of all possible codes. As we have seen earlier in chapter 3, there is an astronomical number of such equivalent codes. This is one of the differences between the neutral space of genetic codes and the puny neutral space of DNA alphabets. There are even more than  $10^{60}$  valid codes that have the same redundancy pattern as the nearly universal genetic code of today's organisms (3 amino acids to which 6 codons are assigned, 8 amino acids with 4 assigned codons, etc.). This is not to say that the code space is completely filled by valid codes. For example, the astronomical number of codes that encode only 19 amino acids are invalid, as are all codes that have no termination signal. Beyond the statement that both the number of valid and invalid codes is astronomical, we know little about the structure of this code space.

The universal genetic code is a good candidate example for mutational robustness that is an adaptation to mutations or to nongenetic change and that has evolved in response to the detrimental effects of such change. As I discussed in chapter 3, the universal code is more robust to mutations and translational errors than the vast majority of alternative codes. That is, in the universal code, random nucleotide changes in a gene are much more likely to cause a replacement of an amino acid in the encoded protein with a chemically similar amino acid than in most alternative codes.

This means that the universal code renders protein structure robust to random nucleotide changes in DNA. Earlier, I discussed evidence that this is not simply an accidental by-product of code evolution. Instead, it results from the natural selection of robust codes. Evolved code robustness means that all valid genetic codes, although equivalent with respect to their coding potential, are not equivalent with respect to another feature: robustness. This points to a theme we will encounter repeatedly. Within a neutral space, such as that of valid genetic codes, robustness may differ greatly among different points or regions. Evolution of the genetic code, which largely occurred in the distant past, occurred within the neutral space of valid codes and led to a code with increased robustness.

These observations imply two general conditions for the evolvability of robustness in any biological system. First, its neutral space needs to contain points or regions with higher robustness. Second, these regions must be reachable from regions of lower robustness during evolution. Both conditions are clearly met for the genetic code. But was the code's evolution unconstrained, that is, did evolution explore all regions of the neutral space of valid codes? The limited cases of recent code evolution (295) suggest that the genetic code may presently evolve through changing assignments of individual codons. If occurring at equal rates at all codons, this process could, in principle, explore all or a large fraction of code space. In contrast, evidence I discussed earlier suggests that early in life's evolution the genetic code coevolved with the biosynthetic pathways of amino acids. That is, in the early, formative stages of code evolution, only a small subset of codes—never all possible codes—were explored. (Despite this constraint, the universal code is more robust than alternative codes within this subset of codes.) In more general terms, the processes generating genetic change, such as change in the structure of genetic codes, may seriously constrain how evolution explores a neutral space, and which regions of the space it explores. Whether robustness of a genetic system can evolve is thus strictly speaking a question about not only the structure of a neutral space, but also how genetic change occurs in it.

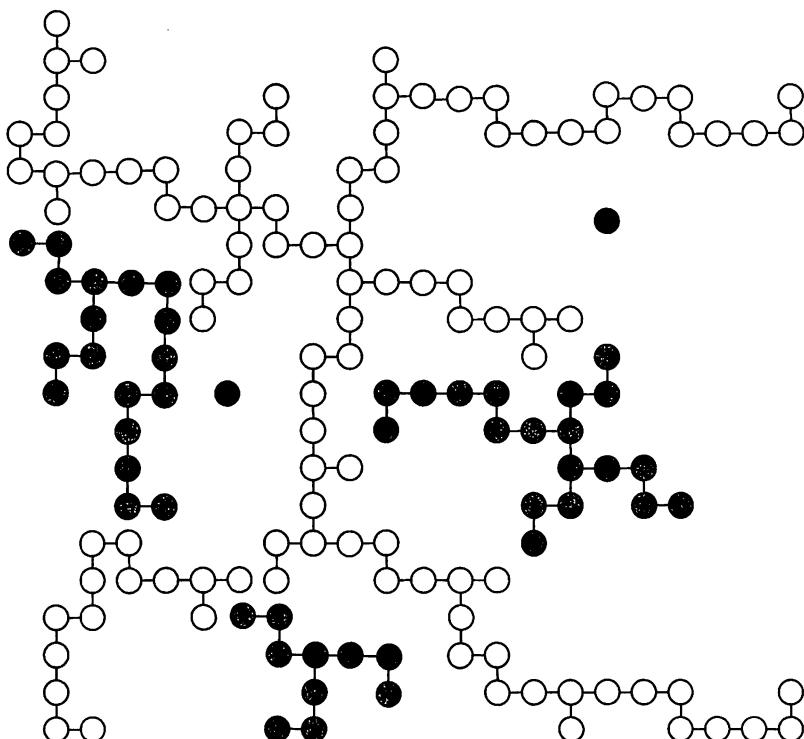
## RNA and Protein Structure

Most work on robustness in RNA and proteins addresses structural robustness to single nucleotide or amino acid changes. Robustness in RNA structure is the best-studied example of robustness in any biological system, but most current insights rely heavily on computational work and a small number of empirical studies. In contrast, empirical evidence for mutational robustness is abundant for proteins, but many questions that

elude current experimentation are unanswered for proteins, because pertinent computational work is comparatively sparse. The following highlights some pertinent points common to both proteins and RNA, points that I discussed separately in chapters 4 and 5.

The relevant space for both RNA and protein molecules is sequence space. A molecule of  $n$  amino acids or nucleotides corresponds to a point in an  $n$ -dimensional space. The  $i$ th axis, dimension, or coordinate of this point has a value that corresponds to the nucleotide or amino acid at the  $i$ th position of the molecule. Again, this space is discrete and  $n$ -dimensional.

RNA and protein structures—minimum free energy structures, to be precise—are extremely unevenly distributed in sequence space. That is, the vast majority of structures are realized by very few sequences. Such structures are also called rare structures. Conversely, a few structures are realized by an astronomical number of sequences (Figure 13.2). Such structures are also called frequent structures. For any one structure, I call the set of sequences that have the structure as their minimum free energy structure the neutral space associated with this structure. This neutral space is the collection of sequences that solve the same biological problem, how to fold into a particular structure, a structure that may be essential for various biological processes. For RNA molecules, this concept is closely related and derived from the concept of a neutral network, which was first coined by Schuster, Fontana, and collaborators (491). These investigators discovered a very important property of RNA sequence spaces: Any pair of sequences folding into a very frequent RNA structure can be connected by a series of single nucleotide changes, such that all intermediate sequences adopt the same structure (Figure 13.2). In this sense, the sequences form a connected network, hence the name neutral network. While the notion of a neutral network is evocative and perfectly suitable for frequent RNA structures, I favor the notion of a neutral *space* for the broader class of systems I discuss here. The first reason is that only sequences folding into the most frequent structures are connected in a neutral network. In contrast, pairs of sequences folding into a rare structure are not always connected through single nucleotide changes (Figure 13.2). Put differently, the set of sequences folding into the same structure can consist of several disconnected subsets for all but the most frequent structures. Hence, these sets of sequences do not form one network. A second reason to favor the notion of a neutral space is that the term neutral network is most appropriate for discrete spaces, such as sequence space, where individual sequences are the nodes of the network. However, other systems, including metabolic pathways and gene regulation networks, may be better represented in a continuous space, such as a space of continuously varying enzyme activities or regulatory



**Figure 13.2** Schematic illustration of three different classes of RNA or protein structures. The rectangular area symbolizes sequence space. Circles correspond to individual sequences in this space, and circles with the same shading correspond to sequences folding into the same (secondary or tertiary) structure. The network of circles shaded in light gray corresponds to a highly frequent structure, a structure realized by many sequences. All sequences folding into this highly frequent structure form a connected, neutral network (491). The three groups of circles shaded in dark gray correspond to sequences folding into the same moderately frequent structure. The sequences folding into this structure do not form a connected network, but instead form three disjoint sets of sequences. Finally, the two black circles correspond to a rare structure, a structure realized by only two sequences that occur at different points in sequence space. The image is misleading in that the actual sequence space is high-dimensional, not two dimensional as suggested by the box.

interaction strengths. The notion of a network does not apply very naturally to such continuous spaces.

As has been most rigorously shown for RNA structures (456), the frequency of a structure is intimately related to its robustness against mutations. The more frequent a structure is, the more sequences fold into

it, and the more neutral neighbors—sequences differing at only one position—the average sequence folding into the structure has. In other words, mutations are least likely to affect the structure into which a sequence folds if this structure is a frequent structure. Frequent structures are most robust to mutations, whereas rare structures are most fragile.

In addition, as in the above case of equivalent genetic codes, the neutral space associated with one frequent sequence is not homogeneous, although the nature of its inhomogeneity is different. Some sequences have many more neutral neighbors than others. Large populations of molecules that all fold into the same frequent structure, and that evolve under the influence of mutations and selection to maintain this structure, can “discover” regions with high mutational robustness in a structure’s neutral space. In other words, their robustness to mutations can increase. The amount by which it can increase is only a property of the structure’s neutral space. That is, this amount is, to a first approximation, independent of factors such as mutation rates and population sizes—as long as they are large—and can be determined from the structure of a neutral space alone (chapters 4 and 16). This finding underscores again how important a system’s neutral space is to understand limits on its robustness and the evolution of robustness.

Robustness in proteins and RNA *can* increase in evolution. But does it? This is a completely different question and it remains largely unanswered. The only empirical evidence is most tentative: Conserved and thus functionally important secondary structures of RNA viruses are slightly more robust than nonconserved structures (chapter 4). To answer this question more systematically and for a broad spectrum of RNA molecules with different biological function, we will need to understand how their evolution is constrained.

Evolution of robustness in the genetic code already illustrated an important kind of evolutionary constraint: constraint by history. Because the genetic code coevolved with pathways of amino acid biosynthesis, evolution did not explore the whole neutral space of valid codes. Proteins and RNA illustrate a second type of constraint, constraint by function. At the root of this constraint is the coupling between mutational robustness and thermodynamic stability: The more robust an RNA or protein molecule is, the greater its thermodynamic stability. Thus, to evolve maximal robustness means to evolve maximal thermodynamic stability. Is maximal thermodynamic stability a desirable feature? Not always. A favorable structure needs to be stable enough such that a molecule can fold into it rapidly and spend an appreciable amount of time in it. However, excessive stability may often be detrimental. For example, important viral RNA secondary structures need to unfold during replication. RNA molecules with the highest possible thermodynamic stability—and thus robustness—may thus slow down the process of genome replication. Metabolic enzymes

provide another cautionary example. Although stably folding into one structure, they need to be flexible enough to adopt minor structural changes necessary to catalyze chemical reactions. Put differently, they need to be optimally instead of maximally stable. Just like for the genetic code, the evolution of protein and RNA sequences is thus constrained to parts of a structure's neutral space, but for different reasons. Sequences in some regions of this space may have properties, such as excessive thermodynamic stability, that may be unfavorable. In this sense, evolution may be confined to regions of a neutral space for functional reasons, and can attain the maximally possible robustness only within these regions.

Hidden behind these observations are two problems that I will later revisit in greater detail: How does robustness evolve in the face of trade-offs with other properties of a system (chapter 18)? And how should we best think of the notion of neutrality, if two systems are the same in some respects but not in others (chapter 14)?

A few remarks about proteins' robustness to recombination will close this section. We saw in chapter 6 that recombination even between distantly related proteins of the same function often preserves this function. In other words, proteins can be highly robust to recombination. The relevant neutral space is that of a protein's structure and function, but the mechanism of genetic change is different. Specifically, recombination transforms sequences not into one of their neighbors in sequence space, but into much more distant sequences. Consequently, evolution, when driven by recombination rather than by mutation, would also explore this space in a different way.

Studies on model proteins suggest that their robustness to recombination is a generic property of neutral spaces for frequent protein structures. However, beyond this insight, we have no explanation for the features of protein sequence space that endow it with such robustness. Also completely unknown is whether such robustness can increase in evolution. That is, can protein sequences exposed repeatedly to recombination come to reside in regions of a neutral space where recombination leaves structure and function unchanged? Again, this is ultimately a question about the structure of neutral spaces.

## Neutral Spaces of Gene Expression

The higher one climbs in the hierarchy of organismal organization, the less information about the robustness of biological systems is provided by systematic experimental and computational perturbations. Instead, one has to rely increasingly on a limited proxy for perturbations, namely evolutionary comparisons of systems that perform the same function in different ways.

While not strictly proving robustness of any one system, such comparisons can suggest that there are multiple different ways of solving the same problem, and thus a large neutral space associated with the problem.

Analyses of gene expression illustrate both the insights one can get from evolutionary comparisons and their limitations. In eukaryotes multiple transcriptional regulators cooperate to ensure gene expression at the right time and place, which is necessary for proper functioning of cellular processes, organ systems, or development. Recall from chapter 7 the example of *even-skipped* gene expression in the fruit fly *Drosophila*. The timing and spatial pattern of *even-skipped* expression is critical for fly development. Different species in the genus *Drosophila* achieve proper *even-skipped* expression through transcriptional regulatory regions with drastically different organization. In some species, binding sites of some transcriptional regulators have all but disappeared. In other species, new binding sites have arisen. However, despite such reorganizations of the *even-skipped* regulatory region, *even-skipped* gene expression is unchanged. Hints of similar phenomena come from a variety of other studies in different organisms (chapter 7).

The concept of a neutral space, analogously to that of proteins and RNA, can be applied to regulatory regions and their evolution. Consider all the DNA in the vicinity of a gene that could potentially influence a gene's expression. This DNA forms a space of regulatory regions whose dimension (number of nucleotides) can be huge. Among all sequences in this space, those ensuring the correct spatiotemporal expression of a gene like *even-skipped* constitute a neutral space.

Several now already familiar questions—currently with no answers—arise for this neutral space. Are there some biologically important gene expression patterns that are realized by few regulatory regions—a small neutral space—and that are thus intrinsically fragile to mutations? Do the majority of biologically realized gene expression patterns have a large neutral space, and can they be realized by robust regulatory regions? If so, can robustness increase in evolution, i.e., can an evolving population of organisms come to reside in a region of this neutral space, where genetic change is especially likely to be neutral? Only computational analyses or a combination of experimental and evolutionary studies could hope to answer these questions.

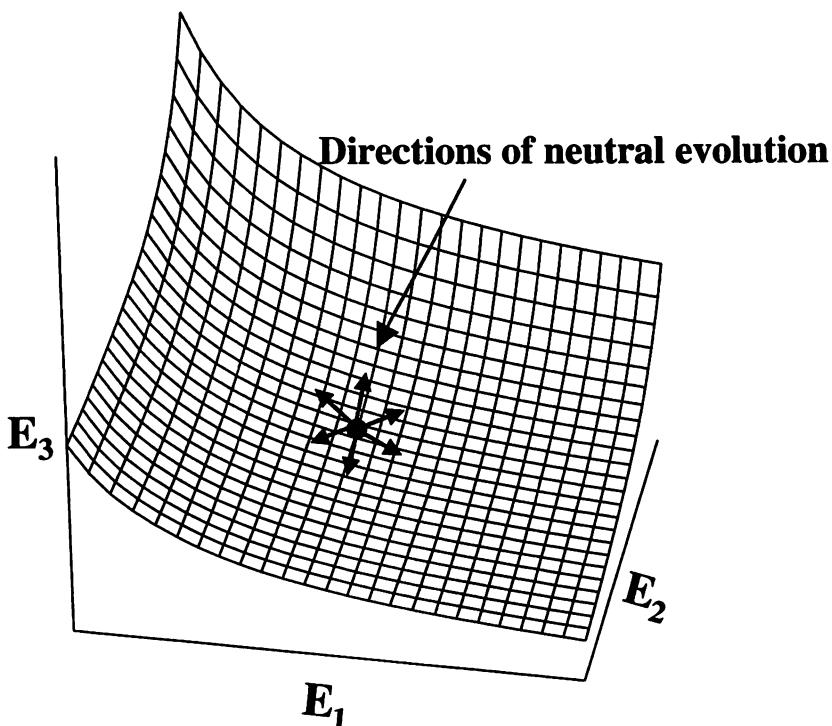
## Metabolic Pathways and Networks

In chapters 8 and 9 on metabolic systems, I reviewed two very different representations of metabolism. The first is a detailed representation of chemical reaction chains—metabolic pathways—that produce biologically

important chemical compounds. This “metabolic control” representation needs information about the activity of individual enzymes. It has been used to show that metabolic pathways can be highly robust to changes in individual enzyme activities. Specifically, the steady-state flux through a pathway—the rate at which substrates are converted into products—may vary little even if the activities of individual enzymes vary enormously. The second, “flux balance” representation of metabolic systems applies to complex reaction networks that may contain thousands of reactions with multiple branch points and cycles. It requires only minimal information, information on the stoichiometry of each chemical reaction in a network. This information is sufficient to determine chemically allowable rates of material flux through each reaction in the network. In addition, among such allowable fluxes, one can identify those fluxes that maximize some desired property, such as the optimal growth flux of a cell. This is the flux that produces all molecular building blocks necessary for the production of biomass in optimal proportions.

These two very different representations of metabolism are necessary because our mathematical models and empirical knowledge about metabolism are limited. The mathematical apparatus of metabolic control representations becomes cumbersome for metabolic networks of even moderate size and few branch points. Moreover, the required information on enzyme activities is simply not available for large metabolic networks. Fortunately for my purpose, the two classes of representations have related neutral space representations. For the metabolic control models, the steady-state flux through a pathway is a function of the enzyme activities  $E_i$  in the pathway. One can thus think of a space of all possible steady-state fluxes  $F$  that can be realized by a linear chain of  $n$  enzymes. This is an  $n$ -dimensional continuous space whose axes correspond to the activities  $E_i$  of these enzymes. For any given steady-state flux  $F$ , there are infinitely many combinations of enzyme activities that can realize this flux. These combinations constitute the neutral space associated with  $F$ . In the simplest case of an unbranched pathway, the enzyme activities resulting in the same flux can be calculated with equation 8.3. Figure 13.3 illustrates the structure of the resulting neutral space for the low-dimensional case of a three-enzyme pathway.

Is this continuous representation of flux space sensible? Because genes encode enzymes, and because each gene has only a finite and discrete number of possible variants, any enzyme has only a finite number of possible activities. Thus, one could argue in favor of a discrete representation of flux space. However, several good reasons exist to prefer a continuous representation. First, because the possible number of alleles at each gene is astronomically large, the resulting enzyme activities can assume many different values, making the continuous approximation unproblematic.



**Figure 13.3** A surface of constant steady-state flux through a pathway of three enzymes. A change in enzyme activities along the direction of the arrows does not change flux and is thus neutral. The surface was determined using equation 8.3.

Second, the mathematical apparatus of chemical kinetics, which ultimately explains the properties of these pathways, is based on continuous quantities such as fluxes and reaction rates. Representing metabolism in terms of enzyme activities and fluxes as elementary and continuous variables is thus simply more parsimonious.

The neutral space associated with a given metabolic flux is neither empty nor small. To use the caricature of Figure 13.3 for the flux space of metabolic control, it is clear that one can change enzyme activities in any direction along the surface of Figure 13.3 without changing the overall flux. One might argue, however, that mutations in individual genes will usually change the activity of one enzyme and will thus displace enzyme activities not along the surface shown in Figure 13.3, but along one of the axes of flux space. Does that mean that mutations in enzyme-coding genes will always have deleterious effects? No. The reason is that flux control is exponentially distributed in multienzyme pathways (chapter 8).

That is, changes in activities of most enzymes change the flux very little, whereas a few enzymes affect flux profoundly. In terms of Figure 13.3, this means that along one or a few axes or enzyme activities, metabolic systems are close to the origin of flux space, implying sensitivity of flux to the corresponding enzyme activities. Along most axes or enzyme activities, however, they are very far removed from the origin, implying insensitivity. Changing the activity of any one such enzyme by a given amount will affect flux by a much smaller amount. Even drastic mutations in enzyme-coding genes on any of these insensitive axes can be effectively neutral, because they change flux by an amount so small that natural selection cannot “see” this change. (I give a more precise definition of “effectively neutral” below.) What we still know very little about is the more realistic scenario where multiple gene loci show variation in enzyme activities among individuals of a population. How do such evolving populations drift through the high-dimensional neutral space of an optimal pathway flux?

The second, flux balance representation of metabolism, also leads to a continuous and high-dimensional neutral space. Define first an  $n$ -dimensional space, each of whose axes correspond to the flux through one of the chemical reactions in the network (*not* to enzyme activity, as in the above representation). Within this space, only some combinations of fluxes are chemically realizable in a steady state, and only some of these fluxes maximize a biologically important property. For example, all fluxes that lead to optimal growth and maximal biomass production form the neutral space associated with the optimal growth flux. These fluxes would constitute the neutral space of maximal biomass production. The neutral spaces of the two representations differ in one important respect. In the metabolic control representation, steady-state flux through a pathway is a function of enzyme activity, completely determined by it. In contrast, the space of the flux balance representation concerns only chemically allowed steady-state fluxes that produce a desirable metabolic output. Whether an organism’s complement of enzymes can achieve the desired fluxes is a completely different matter. However, laboratory evolution experiments with *E. coli* suggest that this capability—if a cell does not already have it—can evolve very rapidly (249).

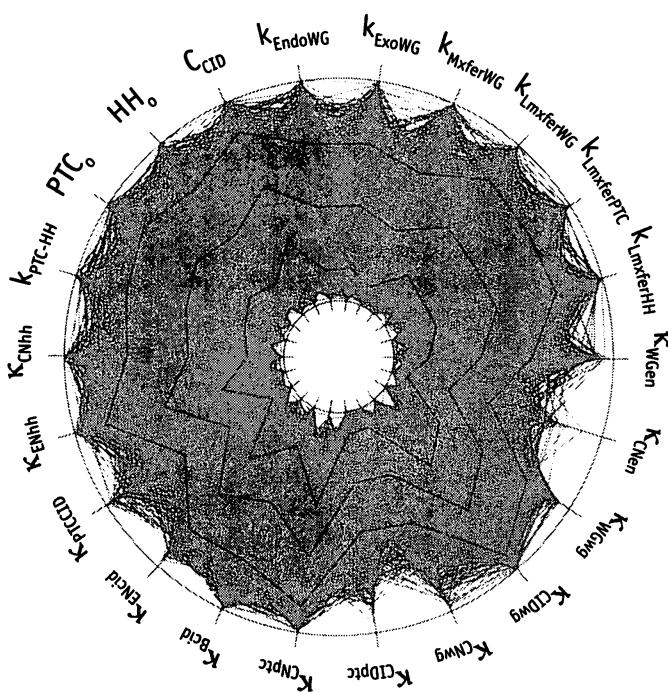
Analyses of optimal growth flux in the flux-balance representation of microbial metabolism arrive at a conclusion similar to that for the metabolic control representation: The neutral space of optimal growth is not empty but vast. Unfortunately, a simple mathematical expression like equation 8.3 for the metabolic control representation does not exist for the complex structure of this neutral space. Pertinent work thus determines numerically how flux can be changed through individual reactions without reducing the growth rate of a cell population. The results are striking.

Recall from chapter 9 that many of the most central chemical reactions of *E. coli* metabolism can be eliminated completely without affecting cell growth by more than 5%. More than 80% of the reactions can be eliminated without abolishing growth, and the flux through most of the remaining essential reactions can be reduced by more than 80% without affecting growth (131–133). Such findings strongly suggest that even a property as important as bacterial growth can be highly insensitive to changes in fluxes through individual reactions in a large metabolic network.

## Regulatory Gene Networks in Development

Chapter 10 discussed a detailed analysis of a genetic network necessary to subdivide a fruit fly's body into multiple segments. This analysis used empirically validated interactions of genes and their products to establish a qualitative model ("who interacts with whom") of the network driving segment polarity gene expression in the fruit fly. To reproduce segment polarity gene expression, the model needs some 50 parameters—most of which have never been measured—that specify factors such as the binding strength of ligands to their receptors, and the expression level of network genes. To bypass the problem these unknown parameters pose, von Dassow and collaborators (571, 572) asked what fraction of randomly chosen parameter values yield a functional network, a network that reproduces the empirically observed expression pattern of segment polarity genes. The answer is striking: For any one parameter, almost 90% of randomly chosen values yield a functional network. In addition, starting from a functional network, many individual parameters can be varied across more than one order of magnitude without affecting network function. Moreover, network function is highly robust to duplications of individual network genes, loss-of-function mutations of network genes that affect only one of two gene copies, and changes in expression states of some segment polarity genes, changes that might be caused by mutations outside the network.

It is straightforward to define the space in which this and other gene networks exist. It is a continuous space, each of whose axes corresponds to one of the parameter values necessary to fully characterize this network. The neutral space of optimal segment polarity gene expression is simply the set of parameter values that yield a working network, as again illustrated in Figure 13.4. This neutral space is vast. Along every one of its axes, a wide range of parameter values are permissible. What the above analysis does not show is whether the actual *Drosophila* segment polarity network resides in a special place in this space, a place more or less robust to mutations than other places. The answer is not known,



**Figure 13.4** “Wheel plot” of parameter combinations that yield a functional segment polarity gene network. Each spoke of the wheel represents one parameter (only 22 out of 48 parameters are shown), and each position along one spoke represents one value within the allowable range (typically three orders of magnitude) for the parameter. Gray polygons correspond to parameter combinations that yield functional networks. Note that for any one spoke, parameter values that yield functional networks are distributed widely over the allowed range. Black polygons represent the mean (middle)  $\pm$  one standard deviation (outer and inner) of such parameter values. See the legend to Figure 10.6 for an explanation of symbols. From Figure 2a in (571) by permission of the Nature Publishing Group.

simply because it would require a precise quantitative understanding of all gene interactions in the network.

Indirect evidence from a variety of other studies speaks to the last question, but not for characters as basic as fly segmentation (chapter 11). Instead, the evidence regards a much wider spectrum of fruit fly characters such as eye morphology, wing shape, and leg architecture, as well as various traits in other organisms. The gene networks that are involved in the development of such characters are poorly characterized. Therefore, we know next to nothing about the structure of the neutral spaces for any of

these traits. However, a string of experiments originating in the 1950s (575) show that these genetic networks—whatever their structure—occupy an especially robust place in a neutral space of proper character development.

The relevant experiments do not systematically perturb genes in these networks. To do so would hardly be possible given that most of these genes are unknown. Instead, these experiments show that natural populations of flies and other organism harbor much cryptic developmental variation. Such variation consists of mutations persisting in a population, mutations in genes responsible for character development that are not normally visible on the character level. As a result, these characters show little phenotypic variation in natural populations. However, when one introduces suitably chosen genes into a population, such cryptic genetic variation reveals itself as an explosion of phenotypic variation. The most striking of these genes encode defective variants of the heat-shock protein Hsp90. This protein interacts with at least 80 other proteins in a variety of signal transduction pathways necessary for development. To impair this protein in a population reveals a wide spectrum of developmental abnormalities in many different characters, abnormalities for which the formerly cryptic genetic variation in genes is responsible. Thus, by manipulating key genes, one can move a genetic network to a region of a neutral space that is less robust to mutations in the form of preexisting genetic variation. This type of analysis shows that mutational robustness of developmental gene networks is under genetic control and thus evolvable. The same holds for other gene networks, including those responsible for some genetic diseases I discussed in chapter 11.

### A Principle of Network Organization Ensuring Large Neutral Spaces

Are there general principles of robust network organization that apply not only to the segment polarity network—one well-understood network among thousands—but to regulatory gene networks in general? One candidate principle among others (36, 175, 288, 480, 481) is that of cooperativity. It is a common feature of regulatory processes such as transcriptional regulation and ligand–receptor interactions. A molecule whose activity is regulated cooperatively by one or more regulatory molecules does not change its activity over a wide range of regulator concentrations. However, it changes its activity drastically, sometimes switch-like, within a small, critical window of regulator concentrations (Figure 10.3). The segment polarity network and other regulatory networks contain many cooperative interactions among their member genes. As I discussed in

chapter 10, models of genetic networks designed to capture cooperative interactions among their member genes show high robustness to changes in the strength of interactions among these genes. Not only that, robustness to mutations changing these interactions is evolvable in such networks. That is, when subjecting these networks to selection to maintain a gene expression pattern, they evolve increased robustness to mutations. This means that the neutral space of this optimal gene expression pattern is heterogeneous and contains regions of high robustness. In addition, impairing the function of individual genes in such models can have effects analogous to the experimental manipulation of genes like that encoding Hsp90: It may render a network less robust to mutations (40).

In sum, regulatory gene networks with cooperative interactions share features with the well-characterized segment polarity network, experimental manipulations of robustness in developmental gene networks, and related phenomena we encountered on lower levels of biological organization. The problems they solve can have large associated neutral spaces leading to high robustness to mutations. In addition, some regions in these spaces show higher robustness to mutations than others. All this is not to say that cooperativity is the only principle of robust network design. Some other principles, such as feedback control (36, 175, 251a, 480), are well known, yet others may await discovery.

## The Building of Organs and Organisms

The highest level of organismal organization is that of whole, many-celled organisms with their tissues, organs, and body plans. Chapter 12 discussed various examples that hint at robustness also on these levels of organization. One of these examples regarded variation in vulval development among various nematode worm species. Specifically, closely related worm species show significant developmental variation even on the small scale of the dozen or so cells necessary to form their vulva. In some species, the gonad is absolutely necessary if all vulval precursor cells are to adopt their proper fate; in others, the gonad is dispensable for some such cells; some worm species require a specific gonadal cell, the anchor cell, for proper vulva formation, whereas others can dispense with this cell; how the anchor cell itself is formed varies among different worm species; the number and cell division patterns of the vulval precursor cells also varies widely; and so on.

Two further, even more striking examples regarded variation in the making of an entire organism among closely related sea urchin and wasp species. Recall that within little more than the time it took humans to diverge from chimpanzees, sea urchin species can switch from indirect to

direct development, completely skipping a complex free-living larval stage. The concomitant developmental changes are radical and include altered nutrient storage capabilities of the egg, a transition from unequal to equal cleavage, and a complete transformation in the map of cell fates in the 32-stage embryo. Yet the final outcome of development, the adult body plan, is essentially the same. Similarly, endoparasitic wasps differ from some closely related ectoparasitic wasps in key aspects of development. They have adopted yolk-poor eggs, total cleavage, and early separation of nuclei by cell walls. Most strikingly, some of them took very little time to adopt polyembryony, where one fertilized egg develops not into one adult but into up to 2000 adult wasps. Concomitant developmental changes range from the highest level of organization—the polarity of the embryo—to the lowest, including expression changes of key developmental genes. But despite such pervasive changes, adults with the same body plan emerge.

The theme underlying these examples is the same. Evolution has rapidly found different—sometimes radically different—ways to build the same organ or the same body. These examples only hint at robustness because they regard variation in development among different species, not perturbations of development in one species. Yet they indicate that at least some mutations that change embryonic development in these systems, albeit in unknown genes, do not affect the final outcome of development.

The neutral space concept can still capture some of this developmental variation. Take variation in cell division patterns among the six vulval precursor cells. Ideally, one would like to codify such variation in terms of the responsible genes and their products. However, these genes may number in the thousands and are largely unknown. It may thus be most parsimonious to represent the space of vulva formation on the level at which it is studied, that of individual cells and their interactions. Define thus a six-dimensional discrete space, each of whose axes represents one of the six vulval precursor cells. To any one worm species assign a coordinate at each axis of this space, a coordinate that corresponds to the number and pattern of cell divisions the respective vulval precursor cell undergoes. (No vulval precursor cell undergoes more than seven cell divisions before the adult vulva is formed, such that one does not have to contend with an astronomical number of possibilities.) All points in this space that lead to a functional vulva comprise the neutral space of vulva formation.

This concludes our tour through the case studies of previous chapters, a tour that pointed out how the neutral space concept can unify the superficially disparate features of many examples from earlier chapters. Before summarizing how neutral spaces can help explain robustness of many systems on all levels of organismal organization, I will briefly discuss some limitations and practical problems with the neutral space concept.

## The Spatial Metaphor and Its Limitations

Spatial metaphors like adaptive landscapes and the morphospaces studied by paleontologists (526) have a successful history in biology. Yet all metaphors have their limitations, and that of a neutral space is no exception. The last examples from organismal development point to these limitations. We have not found an elegant and concise spatial way to codify all successful (and unsuccessful) ways to build a body. It is thus no surprise that all equivalent ways of making the same body—ways that produce the same adult body plan—elude a spatial representation as well. The radical changes—transformed fate maps, cleavage patterns, polyembryony—associated with some changes in development would make a spatial representation certainly unwieldy, and perhaps impossible.

These limitations are not restricted to the highest level of organismal organization. They become obvious also on other levels of organization whenever evolution changes a system's organization too drastically. Examples include the addition of new genes to a genetic network; the addition of new pathways to a metabolic network; change of protein sequences through unequal recombination, which can change not only the length but also the structure of a protein; changes from a genetic code based on nonoverlapping nucleotide triplets to some other coding scheme; or genetic alphabets that do not use Watson-Crick pairing to transmit genetic information. Mathematical analyses suggest that these limitations of spatial metaphors are not just the result of our limited imagination (518). Instead the spaces I considered here—metric spaces, to be precise—have, like any concept, fundamental limitations in capturing evolutionary change and innovation. The neutral space metaphor is thus most useful when comparing systems whose fundamental architecture is the same, but whose parts may interact differently.

A second limitation stems not so much from the spatial metaphor itself but from our intuitions about space. Because we perceive the world three dimensionally, we tend to think about any space in such terms. Yet most of the spaces we encountered here are high dimensional, and some of them are not even continuous but discrete. Our imagination's limitation to three-dimensional, continuous spaces impairs our thinking not only about neutral spaces, but about any other spatial metaphor, such as the classical evolutionary metaphor of an adaptive landscape (632). Adaptive landscapes, which represent the fitness of a biological system as a function of its properties, bear a close relationship to neutral spaces. In fact, every neutral space we have encountered here corresponds to the peaks of a multi-dimensional adaptive landscape. In some cases, such as the neutral spaces of protein structure, these peaks can form elaborate

connected networks that permit an almost indefinite explorations of a vast sequence space. However, this topology of peaks is impossible to capture in a three-dimensional landscape with its well-separated peaks and valleys.

### How Neutral Is Neutral Enough?

Systems whose performance can be measured in terms of continuous quantities, such as metabolic flux, pose special problems to evaluate robustness. Earlier, I mentioned an example where a greater than 80% reduction in the flux through one essential chemical reaction would reduce the growth rate of a bacterial cell population by less than 1%. From an engineer's standpoint, any system that does not fail catastrophically in the face of such drastic change may be an impressive feat. Natural selection, however, can be a much harsher judge. A tiny reduction in fitness may lead to elimination of a mutant from a population within a few hundred generations. However, natural selection cannot resolve infinitely small fitness differences. Because of random sampling effects—random genetic drift—as individuals reproduce from one generation to the next, mutants with small fitness differences to a wild-type gene may escape natural selection (222). Population genetics theory makes a simple and well-corroborated prediction of the conditions under which this occurs. Let  $s$  denote the fitness reduction a mutant suffers and  $N_e$  the effective size of an evolving population. ( $N_e$  is a number that reflects not only the number  $N$  of individuals in a population but also various of their life history features.  $N_e$  can be determined from  $N$  (222).) Mutations that cause a fitness change of much less than  $s = 1/4N$  are “effectively neutral,” a notion introduced by Motoo Kimura (284). That is, natural selection does not eliminate such mutations. They can persist indefinitely in a population. Thus, the question of whether a 1% reduction in metabolic output is disastrous or effectively neutral has no general answer. It depends on the size of an evolving population. For evolving populations of constant size  $N_e$ , one can replace the notion of a neutral space with that of an effectively neutral space.

### Neutral Spaces and Robustness

At first inspection, the pervasive robustness we see at many different levels of biological organization—from the genetic code to genetic networks—may seem mysterious. However, its explanation is contained in the structure of neutral spaces and how evolution finds solutions to biological

problems. To recapitulate, among all possible ways—good or poor—to solve a biological problem, a neutral space is the subspace of all equivalently optimal solutions. With this definition in mind, I now outline and summarize how neutral spaces can help understand why robustness is so pervasive in biological systems on all levels of organization.

Neutral spaces can help explain two different evolutionary origins of robustness in biological systems. I illustrate the first of them with a hypothetical example. Assume an organism's survival depends on finding—through a blind evolutionary search in sequence space—a biological catalyst (protein or RNA) able to promote a specific chemical reaction. Many of the possible tertiary structures a protein or RNA molecule could adopt will be outright incompatible with the mechanism of the chemical reactions, but some structures will be able to catalyze the reaction. These structures may not resemble one another. For instance, comparisons of real enzymes with the same function from different organisms indicate that such enzymes can have vastly different tertiary structures. And because tertiary structures are unevenly distributed in sequence space—some are frequent, others are rare—the subset of tertiary structures catalyzing any one reaction is likely to have the same property. This leads to the key question: Which kind of structure able to catalyze a reaction—frequent or rare—will a blind evolutionary search of sequence space turn up? A frequent structure, of course. (Protein engineers also call such structures designable structures.) And the frequent structures are also the structures most robust to mutations.

Although we know less about the connectivity of other neutral spaces, I suggest that this is a general principle that applies on all levels of organismal organization, from genetic information carriers to gene networks comprising thousands of genes.

Evolutionary searches, which grope blindly in a space of possible solutions to a problem, will most likely find frequent solutions, solutions realized by many variants of the same system. These are solutions with a large neutral space, solutions that can be realized by robust biological systems.

This statement emphasizes that robustness is partly an intrinsic feature of biological systems, a result of how evolution “discovers” solutions to a problem organisms face. Evolution is simply more likely to find robust (frequent) solutions than fragile (rare) solutions.

Actual evolutionary searches are, of course, more complicated than this hypothetical example. For one thing, they usually do not start from scratch, but tinker with systems that already serve some other purpose. However, the same principle applies to these systems and their modifications that successfully solve a new problem. A modification corresponding to a more frequent (robust) solution is more likely to be found than

a modification corresponding to a rare (*fragile*) solution. This may explain why robustness of protein structures tends to increase in the creation of new protein structures through successive random modifications of other, ancestral structures (552a). Incidentally, this explanation also covers one possible origin of the occasionally observed *fragile* biological system (chapter 18), such as a protein whose function can tolerate few or no amino acid changes: Some fragile systems may correspond to rare solutions to a problem, solutions with a neutral space that is small, but that an evolutionary search can nonetheless unearth occasionally.

The second cause of robustness comes from incremental evolution within a neutral space.

The neutral spaces of solutions to many biological problems contain some regions of low robustness and others of high robustness. Through evolution by natural selection, some biological systems have come to reside in regions of high robustness.

Such evolved robustness can be an adaptation to both genetic and nongenetic change (chapters 16 and 17). In systems as different as the genetic code, the codon usage of proteins (444a), and developmental gene networks, we have empirical evidence for such evolved robustness. In others, like protein structures, we know only that robustness is evolvable. Whether it did evolve depends on a variety of factors, such as the historical and functional constraints that a system's evolution is subject to. In sum, I posit that a biological system's robustness (or lack thereof) is explicable by these two factors: whether evolution found a frequent or rare solution to a problem, and whether adaptive evolution toward increased robustness occurred within the neutral space associated with this solution.

# 14

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## Evolvability and Neutral Mutations

This chapter's central question is whether robustness fosters or hinders evolvability. To begin with, what is evolvability? The word "evolvability" has two main usages (101, 183, 287, 445). According to the first of them,

a biological system is evolvable if its properties show heritable genetic variation, and if natural selection can thus change these properties.

A second usage ties evolvability to evolutionary innovations:

a biological system is evolvable if it can acquire novel functions through genetic change, functions that help the organism survive and reproduce.

Functional innovation comes in many different sizes and shapes, from enzymes with new catalytic activities, to novel complex organs such as eyes or wings (390).

The two usages are far from synonymous. Most importantly, not all systems that are evolvable in the first sense are evolvable in the second sense. Consider an enzyme-coding gene that is subject to different mutations in different individuals of a population. These mutations cause the enzyme's activity to fluctuate among different individuals. If such heritable genetic variation affects fitness, perhaps through variations in metabolic flux, then natural selection can change enzyme activity. The enzyme's activity is thus evolvable in the first sense. However, even after millions of years, no mutation might endow this enzyme with a new catalytic activity, an activity perhaps that might permit survival in a completely new environment. Thus, even though it is evolvable in the first sense, the enzyme's activity need not be evolvable in the second sense. The converse, however, does not hold. Every system that is evolvable in the sense of being innovative can evolve by means of natural selection. Put differently, the ability to innovate is the more profound usage of evolvability. It encompasses the first usage and much more. Naturally, we know much less about it.

Robustness is one of several factors that can affect evolvability in either sense (287). A central question is whether robustness fosters or hinders evolvability. It is clear that robustness will not increase evolvability in the first sense. In a highly robust system a given number of mutations will

have smaller phenotypic effects than in a less robust system: Thus, robustness reduces the amount of heritable genetic variation on which selection can act. But, more importantly, does robustness hinder or foster innovation? This is a more difficult question, to which I devote this chapter.

One can adopt two conflicting perspectives on this question. The first arises again from the observation that robustness causes many mutations to be neutral. Neutral mutations, by definition, are invisible to natural selection and can thus not be the source of innovation. Thus, increased robustness means fewer evolutionary innovations.

The second perspective gives neutral mutations a key role in innovation. Although many mutations in a robust system do not change its primary function, they can change other system features, features that harbor the seeds of future evolutionary change. Put differently, a system capable of fulfilling its primary function in many different configurations—explorable through mutation—has sufficient flexibility and degrees of freedom to adopt other features. To use Gould’s term (202) of exaptations—organismal features that may become adaptations only long after they arise—robustness facilitates exaptations. From this perspective, neutral mutations themselves are the key to evolutionary innovation: Robustness implies that many mutations are neutral and such neutrality fosters innovation.

### **What Is a Neutral Mutation?**

A key difference between the two perspectives of the last two paragraphs is their tacit understanding of neutrality. To be able to side with one of them, I must thus first examine this notion. Neutral genetic change, first made popular by Kimura in his neutral theory of molecular evolution (285), is commonly understood as genetic change that does not affect an organism’s fitness. In addition, neutral change has to be neutral in any environment, physiological condition, or genetic background. I will call this the “essentialist” view of neutral change, where being neutral is a property only of a mutation itself—it is part of the “essence” of that mutation—and not of any other factor such as the genetic background.

### **Neutrality: Can It Be Assessed Experimentally?**

These two aspects of neutrality’s definition also encapsulate its biggest problems. First, how can we determine whether a mutation does not affect fitness? Beyond the commonplace that fitness means the ability to survive and reproduce, fitness is difficult to define properly, and nearly

impossible to measure rigorously (333). To give a simple example, laboratory evolution experiments in microbes often use cell division rates of bacterial strains as an indicator of fitness. While growth rate is certainly an important aspect of fitness, a myriad other equally important aspects exist, including survival under starvation conditions, heat-resistance, sporulation efficiency, germination rates, and so on. In addition, growth rates themselves could be measured in countless different laboratory environments. Which of these would be most representative of the environments a microbe encountered in its recent evolutionary past? The answer is usually unknown and perhaps often unknowable. Such problems are exacerbated in higher organisms, where sexual reproduction, age-specific mortality and fertility, an increased ability to change the environment, and smaller population sizes pose daunting principal and technical problems. Taken together, these difficulties mean that an unassailable measurement of any organism's fitness does not exist.

A second approach to identifying neutral mutations applies to well-understood systems inside an organism. For example, assume that you are concerned with the neutrality of a mutation in a mundane gene, such as that encoding the glycolytic enzyme phosphoglucose isomerase. This enzyme interconverts glucose 6-phosphate and fructose 6-phosphate. To determine whether a mutation in its gene is neutral, you could simply measure the mutation's effect on enzyme activity. The approach seems simple enough, but it is doomed to fail. The reason is that many proteins have multiple and unforeseeable biochemical activities or biological functions. Phosphoglucose isomerase itself serves as an example (258). In vertebrates, it is the same protein as neutrophilic, a cytokine causing immune cell maturation and survival of some embryonic spinal nerve cells (78, 145). In addition, phosphoglucose isomerase also serves as autocrine motility factor (600), a cytokine that stimulates cell migration. As if that were not enough, it can also cause differentiation of human myeloid leukemia cells (636). And who knows what other functions await discovery?

Phosphoglucose isomerase is no exception in its multifunctionality. Aminoacyl tRNA synthetases, the enzymes that charge RNAs with amino acids for translation, provide even more striking examples. They can also bind DNA and regulate transcription, bind messenger RNA and regulate translation, participate in the splicing of some messenger RNA, act as co-factors in RNA trafficking, and stimulate chemotaxis of immune cells (359). Among a growing list of further examples (258) is thrombin, a protease that "normally" cleaves fibrinogen to produce fibrin required for blood clotting. Yet it also serves as a ligand for the receptor PAR-1, whose activation causes platelets to adhere to each other (573). To measure changes in well-understood aspects of a protein's function may thus be highly misleading in identifying neutral mutations: One

can simply never be sure of having identified all aspects of a protein's biological function. The same holds of course also for systems on other levels of biological organization. Perhaps the most notable examples come from regulatory gene networks like the segment polarity network. Here, some network genes or the whole network can serve to pattern different body regions at different times in development (189, 280). Taken together, all these examples point to the critical role of the observer or experimentor in asking the right questions of a biological system, questions that yield insight into its function. They also show that we can never be sure that all the right questions have been asked.

### The Evolutionary Approach to Identifying Neutrality

The last paragraphs show that neither fitness nor a biological system's performance—that is, all aspects of it—can be measured in practice. If this is so, then the opening definition of (fitness-centered) neutrality may seem operationally useless. However, the following, time-honored evolutionary approach may provide a detour around this problem. This approach rests on the second aspect of neutrality's definition, namely that a neutral mutation must be neutral regardless of physiological state, environment, or genetic background. The approach takes advantage of a simple yet fundamental population genetic insight: Neutral mutations that occur in a population go to fixation—attain a population frequency of one—at a clocklike and constant rate (285). Importantly, this rate depends only on the rate at which neutral mutations occur, and not on other parameters, such as population sizes. The rate is thus completely independent of the peculiarities of a population's demographic history. This does not apply to mutations whose fate is influenced by natural selection. Specifically, whether such mutations go to fixation may depend on what kind of gene, noncoding DNA, or genetic network they occur in. In addition, it depends on factors such as population sizes and organisms' mating preferences.

A wide variety of tests ask whether this and similar properties hold for the genetic variation that occurs in a population. Such tests can be used to ask whether many (or any) mutations found in a population are neutral. Although these tests have weaknesses, including a frequent lack of statistical power and the possibility of being misled by demographic peculiarities of populations, they are the currently best available approaches to detect neutral mutations (311). These tests compare variation either in nucleotide or amino acid sequences within and between species. Most mutations or alleles that are detectable in a population have moderate to large frequencies and are thus old—at least many generations old, but

often several million years old. Thus, by studying mutations that have arisen a long time ago, such methods essentially average over all the different genetic backgrounds—variation in other parts of the genome—that a mutation may have encountered, and over all the environments to which an organism was exposed. To demonstrate neutrality, these tests thus require that neutrality is an essential feature, in the above sense, of a mutation.

Are there many mutations that behave neutrally according to this prediction? This question became part of the 20th century's neutralist-selectionist debate. The neutralist camp argued that the vast majority of genetic variation observed in natural populations is neutral variation, whereas the selectionist camp argued that much of it is influenced by natural selection. If one had to take score after more than thirty years of debate and data analysis, the selectionists would clearly win by points (128, 310, 312). One of the key insights that emerged from the neutralist-selectionist debate is that even the most paradigmatic candidates for neutral mutations have provided evidence for selection. Some of the best examples are mutations in a gene that change one codon into another codon for the same amino acid. Such synonymous mutations are classical candidate examples of neutral mutations. Yet such mutations can reduce the rate at which a messenger RNA is translated into protein, if they occur toward a codon whose corresponding transfer RNA is sparse in the cell. Thus, synonymous mutations, especially those at genes that need to be highly expressed, are subject to selection (83, 84, 640). In addition, if a gene's optimal expression level changes over time, then the strength of selection on its synonymous mutations may also change.

A plethora of candidate examples for neutral mutations other than synonymous mutations exist. They include mutations in gene-poor parts of the genome, such as telomeric regions and heterochromatin, or mutations in noncoding and nonregulatory DNA. Such candidates for neutral mutations are less-well studied, but they can still serve to illustrate how genetic variation or environmental change could lead to selection acting on neutral mutations. Consider mutations deep in a region of noncoding human heterochromatin, perhaps in a sequence that is a member of the *Alu* family of short, repetitive, interspersed elements (336). Such mutations are classical examples of neutral mutations, mutations in “junk” DNA. However, because genome rearrangements large and small are frequent in many eukaryotes (135), such DNA elements can come to reside in the vicinity of a gene, where previously neutral mutations can affect transcription, translation, or splicing, and thus be all but neutral.

These two classes of examples—synonymous mutations and mutations in noncoding DNA—all regard the dependency of neutrality on genetic background. But what about dependency on environmental change?

Potential examples of this kind of dependency are also numerous, and I cite examples from just two maximally diverse organisms, bacteria and humans. The first example is very simple. Consider a mutation in an enzyme-coding gene that changes a bacterium's ability to extract energy from a carbon source such as gluconate. Such a mutation may not affect fitness in environments dominated by other sugars, but can do so strongly if gluconate is the sole carbon source (223). More generally, many metabolic genes that are dispensable in one environment may be essential in another (247). This notion is consistent with the observation that intracellular parasites, which live in very stable and nutrient-rich environments, shed many metabolic genes that would be essential in free-living organisms.

A second, more complex example regards a human cancer, hereditary paraganglioma type 1. This cancer is caused by mutations in the gene encoding the enzyme succinate dehydrogenase, which is thought to be involved in oxygen sensing (27a). The incidence and severity of this disease varies significantly with elevation. Both are greater in higher elevations with lower oxygen concentrations than in lower elevations with higher oxygen concentrations. In other words, chronic hypoxia is a risk factor for paragangliomas. The population genetics of this disease has been studied comparatively in two human populations living at different average altitudes, one in the Netherlands (low altitude), and another one in the United States (higher altitude) (27a). Affected individuals in the Netherlands typically are descendants of a small number of common ancestors that harbor a disease-causing mutation. In contrast, most affected individuals in the United States harbor different mutations. The available data suggest that at least some alleles associated with this disease can spread through random genetic drift in the Dutch population, because of the lower disease incidence there, but not in the U.S. population, where the disease is more frequent and where natural selection is stronger (27a). In other words, some alleles of succinate dehydrogenase are more likely to be neutral in an oxygen-rich environment than in an oxygen-poor environment.

It takes little imagination to come up with circumstances under which natural selection could favor or eliminate any conceivable mutation that would appear neutral at first glance. With this in mind, it appears much less surprising that studies of molecular evolution—typically averaging over many millennia of genetic and environmental change—suggest that the majority of mutations do not behave neutrally but have been under the influence of natural selection. Equally important is the suggestion—from studies of enzyme polymorphisms—that such selection pressures on mutations are not constant but vary over time (128). A mutation may affect fitness at some times but not at others.

## A Different Perspective on Neutrality

In sum, if one insists on an essentialist, fitness-centered definition of neutrality, then neutral mutations may be extremely rare or nonexistent. The main reason is that one can always conceive of a genetic or environmental change that renders a previously neutral mutation beneficial or detrimental. One may thus be inclined to abandon the concept altogether as practically useless. But what about the many examples of robust systems I discussed here? They include enzymes that can tolerate thousands of amino acid changes, genetic networks that can produce the same gene expression pattern despite widely varying gene interactions, developmental pathways that can buffer much genetic variation, and different cell division and interaction patterns that lead to the formation of the same organ or organism. What to call genetic change that does not affect these systems? “Neutral” is of course a natural choice. However, to use the term in this context requires a radical change in definition.

First, we have to abandon the notion that a neutral change must not ever affect fitness, either now or in the future. As I argued above, this notion is operationally of limited use, and any neutral change can be turned nonneutral through suitable genetic and environmental change. Instead, we should focus on one specific aspect of a system’s function, such as its ability to form a tertiary structure, catalyze a chemical reaction, bind DNA, produce a gene expression pattern, or form an intact organ. (With the help of good biological intuition and luck, we will, of course, study system properties that bear on an organism’s ability to survive or to reproduce.)

Second, we must abandon an essentialist notion of neutrality. The above examples showed the flaws of this notion, and so do some of the mechanistic examples I discussed in earlier chapters. Let me recapitulate a few of them. Figure 4.5 illustrated how genetic change in an RNA molecule can influence the neutrality of a mutation, in the sense that the mutation does not change the molecule’s minimum free energy secondary structure (161). The figure showed an RNA sequence folding into a particular secondary structure, where nucleotide substitutions at some positions are neutral. However, a C → G substitution at one particular position—in and by itself also neutral—changes the number of possible neutral substitutions at other positions. That is, mutations that were previously neutral at some of these positions now alter the secondary structure and are no longer neutral.

A related example from elsewhere in the biological hierarchy is cryptic variation in developmental genes. Neutral variation in these genes is variation that does not perturb the development of complex organs like eyes, wings, and legs. Such variation becomes nonneutral if certain genes mutate,

such as the gene encoding the heat-shock protein Hsp90. The various examples of “monogenic” diseases whose outcome is influenced by multiple genes fall in the same category (chapter 11). Examples of how environmental change can affect neutrality are too numerous to discuss exhaustively, so I will just mention a few examples from metabolic systems. Recall from chapter 9 that each of 14 core metabolic reactions of *Haemophilus influenzae* can be eliminated without substantially affecting growth if the availability of fructose and glutamate is held constant. However, when the environment varies, in the sense that the availability of these two essential compounds changes, 5 of these 14 reactions become essential. Similarly, the sensitivity of photosynthetic activity to the enzyme Rubisco’s (ribulose-1,5-bisphosphate carboxylase-oxygenase) activity in tobacco varies drastically with the lighting conditions under which plants are grown. Finally, variants of many enzymes differ in how sensitive enzyme activity is to changing environmental temperatures.

In sum, it may be best to think of neutral mutations in the following way:

A neutral mutation does not change a well-defined aspect of a biological system’s function in a specific environment and genetic background.

This is no longer an essentialist definition of neutrality: A mutation’s neutrality depends not only on the mutation itself but on its interactions with other genes and the environment. Both may change over time.

I note in passing that the abandonment of essentialist concepts has successful precedents in the history of biology. This point is illustrated by the demise of essentialist species concepts such as the 19th century’s typological species concept (364). Whether an organism belonged to a particular species according to this concept was an essential property of the organism, a property only of one organism’s features. However, because organisms vary greatly in their features within a population, this species concept is of limited use. It was replaced by other species concepts, most notably the biological species concept, which is a nonessentialist concept. The biological species concept centers not on properties of individuals, but on their interactions and on their location, specifically on the ability to reproduce with each other.

What would we gain by adopting this nonessentialist perspective on neutrality? First and most simply, a name, a name for a kind of change that leaves one aspect of a biological system unperturbed. Second, this perspective buys us the ability to make distinctions among different features of a system. While one feature—say, its most stable secondary structure—may be unaffected by a mutation, another feature—such as the thermodynamic stability of this structure—may be affected profoundly. Such distinctions facilitate understanding how neutral change can lead to innovation.

Naturally, changing our perspective on neutrality also has a price. Most importantly, we lose the great generality and conceptual clarity that comes with any essentialist concept. Unfortunately, essentialism is for a simpler world than ours: An essentialist notion of neutrality may not apply to anything. The second price to pay is that we abandon the tight linkage between neutrality and fitness. But it was precisely this linkage that rendered fitness–neutrality of questionable value: Measuring the fitness effect of any one aspect of a system’s function is impossible; and on the long timescales of molecular evolution studies, most mutations are not fitness-neutral. Thirdly and relatedly, the nonessentialist concept of neutrality clearly has limitations and gray areas where its application is awkward. For example, I used the word neutrality when discussing the massive reorganizations of development that do not affect the adult body plans in parasitic wasps and sea urchins (chapter 12). Such changes—ultimately caused by genetic change—are clearly neutral in the above sense, because they do not affect the adult body plan and thus leave key aspects of how the adult interacts with the world around it unchanged. However, they entail many life history changes that are obvious adaptations. Despite an unchanged adult body plan, the endoparasitic mode of development is an adaptation, an adaptation to the relatively benign and nutritious environment inside a host organism. Similarly, the rapid transition from indirect to direct development in sea urchins is associated with multiple adaptive life-history changes.

## Neutrality and Innovation

If we adhere to the traditional, essentialist notion of a neutral mutation, then neutral mutations are irrelevant to innovation and evolvability. If a neutral mutation must not affect fitness under any circumstances, it could not possibly have anything to do with new adaptation. This is at the heart of the perspective that neutrality hinders innovation. However, if we view neutrality as restricted to one aspect of a system, then other (changed) aspects may provide new adaptations or exaptations and neutrality can become key to innovation.

Many examples could be used to illustrate anecdotally how neutral change in this sense could foster innovation (183). Unfortunately, there are few well-studied examples, and most of these come from the molecular level of organization. I will discuss a few examples related to systems I covered previously. They all contain loopholes and are suggestive rather than conclusive. To close these loopholes would be a worthwhile subject of further investigation.

Among pertinent examples I discussed earlier, RNA structure and function is perhaps the most evocative (chapter 4). Computational work shows

how repeated mutations, neutral with respect to RNA secondary structure, can explore sequence space such that new structures—innovations—can become accessible through single mutations. An important experiment by Schultes and Bartel (487) suggests that a similar principle may apply not only to secondary structure—a mere proxy for some aspects of RNA function—but also to biological activities of RNAs, such as the catalytic activities of ribozymes. These authors showed that two ribozymes with radically different tertiary structures and very different catalytic activities can be interconverted by a series of single point mutations. Most of these point mutations do not reduce catalytic activity and are neutral. Some of the intermediate sequences possess both catalytic activities, albeit at reduced rates. This suggests that the robustness of ribozymes to point mutations, even if it does not lead all the way to catalytic innovations, paves the ground for such innovation.

A second class of candidate examples regards the multifunctional proteins for which I mentioned some examples earlier. They include the glycolytic enzyme phosphoglucose isomerase, with a variety of functions as a cell-signaling molecule; thymidine phosphorylase, which catalyzes the dephosphorylation of thymidine and deoxyuridine, and is the same as an endothelial growth factor (178, 221); aconitase, an enzyme in the tricarboxylic acid cycle, which also serves as a translational regulator of ferritin expression (277); carbinolamine dehydratase, which serves in phenylalanine metabolism but also regulates the DNA binding activity of the homeo-domain transcription factor hepatic nuclear factor 1 $\alpha$ . Most significantly, proteins such as phosphoglucose isomerase and thymidine phosphorylase occur both in eukaryotes and in prokaryotes. Their original and still essential enzymatic function thus predates other functions, such as the cell-signaling functions important to many-celled organisms. Have the eukaryotic proteins acquired the ability to carry out these functions after the origin of multicellularity? If so, change neutral with respect to early enzymatic functions may have led to innovations in these proteins.

In some cases, such as crystallins, evolutionary innovations required a tissue-specific increase in gene expression. Crystallins are proteins with a variety of functions that have been co-opted as lens proteins in the eye. In the eye lens their high expression confers the ability to refract light. Many crystallins have undergone gene duplication, but nonduplicated crystallins also exist. They include  $\epsilon$ -crystallin, which is the same as lactate dehydrogenase, and  $\tau$ -crystallin, which is the same as  $\alpha$ -enolase (441, 556). In such nonduplicated crystallins changes in regulatory DNA regions have occurred that allow enhanced gene expression in the lens. Regulatory regions serve as prime examples of how genetic change may be neutral in one respect—gene expression in one tissue—and yet lead to innovation in other tissues. The root cause of such neutrality is the vastness of eukaryotic

regulatory regions. Small islands of transcription factor binding sites are separated by huge swaths of DNA in which mutations can give rise to new binding motifs for transcription factors by chance alone (527). Even in lower eukaryotes such as yeast—which has much smaller regulatory regions than higher eukaryotes—regulatory regions can evolve extremely rapidly. An illustration of this principle is provided by a laboratory evolution experiment, where yeast cells evolved in an environment favoring oxidative metabolism under glucose limitation (151). In merely 250 generations, hundreds of genes, many of them metabolic, evolved stable expression changes in response to this environment.

Candidate examples of neutral change leading to innovation can also be found at the next-higher level of biological organization, that of genetic networks. The genes of the segment polarity gene network I discussed in chapter 10 have highly conserved function and expression patterns and may drive segmentation in all insects. Yet these genes have also been redeployed to pattern organismal features that arose after the insect body plan. An example is the eyespot of butterflies, which is an evolutionary innovation specific to some Lepidoptera and serves to avoid predators. Several segment polarity genes are involved in eyespot formation, where their regulatory interactions are different from those they show during early segmentation (280).

All these examples indicate that biological systems can retain old functions while acquiring new functions. Whether these new functions originated as adaptations or exaptations, that is, whether the new functions originated long after the old ones, remains to be seen. At the very least, however, these examples suggest that change neutral with respect to one aspect of function could lead to innovation in other aspects. For most systems, robustness means that they can harbor a large reservoir of neutral mutations and, as a by-product, a greater potential for innovation. It is, for instance, no coincidence that the evolution of regulatory regions is hailed as the root cause of adaptations as fundamental as those that distinguish humans and chimpanzees. It is a sign of how important neutral change, properly defined, is for innovation.

# 15

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## Redundancy of Parts or Distributed Robustness?

This chapter asks which of two possible *mechanistic* causes of robustness is more important in genetic systems. These two causes are redundancy of a system's parts and distributed robustness, which emerges from the distributed nature of biological systems, where many (and different) parts contribute to system functions.

Much like robustness and evolvability, biologists use the term redundancy in more than one way. One usage invokes redundancy if a gene's activity can be changed or a system's part can be removed without affecting key system properties (306). Another usage refers to redundancy only if two parts of a system perform the same or similar tasks. The two notions are not synonymous. A system may be unaffected by changing or removing a part, yet no two of its parts may have identical functions. Examples include proteins, whose tertiary structure is the result of cooperative folding involving many amino acids. No two amino acids may play identical roles in the folding process, yet protein structure can be highly robust to changes in individual amino acids.

I will use redundancy strictly in the second sense—redundancy of parts. This usage not only is consistent with one of the main dictionary definitions of the word, namely “unnecessary repetition” (3), it is also consistent with the usage of engineers concerned with designing reliable systems (177). In engineering, redundancy is the main pillar of system reliability, partly because it is conceptually straightforward to build systems with redundant parts. But how important is redundancy for robustness in biological systems? Less important than one might think.

### Gene Redundancy

Genes encode the most important parts of biological systems above the gene level—proteins. It is thus no surprise that the role of gene redundancy in robustness of biological systems is receiving great attention. The notion that redundancy of genes might be important for robustness emerged with the ability to generate gene deletion, synthetic null, or gene knock-out strains of various organisms, that is, with the ability to

eliminate specific genes from a genome. Many such gene-deletion experiments yielded surprises. Specifically, they revealed that genes thought to be involved in key biological processes could often be removed without affecting the organism. This phenomenon exists in all organisms and all kinds of genes, be they enzymatic, structural, or regulatory (67, 196, 197, 199, 239, 264, 341, 345, 346, 539, 547, 596, 597).

Although the phenomenon is too pervasive to survey exhaustively, I will discuss a few examples. The *CLN1* gene of budding yeast regulates the activity of the yeast cyclin-dependent kinase Cdc28p (397). Proper regulation of Cdc28p is required for the transition from the G<sub>1</sub>-phase to the S-phase of the cell cycle. However, a null mutant in *CLN1* grows and divides normally on a minimal growth medium (504). The *BarH2* gene from the fruit fly *Drosophila melanogaster* encodes a transcription factor with a homeobox DNA binding domain. It is involved in sensory organ development, yet its deletion does not cause morphological defects in sensory organs (230). The extracellular matrix protein tenascin of mice is thought to play an important role in morphogenesis during embryonic development. Yet the deletion of a gene encoding this protein leads to no detectable developmental abnormalities, to no change in the distribution of other extracellular matrix proteins, and to no detectable reduction of fertility (473). The yeast *HMG2* gene encodes a key enzyme in the sterol biosynthesis pathway, 3-hydroxy-3-methylglutaryl-coenzyme A reductase. The end products of this pathway take part in processes ranging from electron transport to DNA replication. Yet the *HMG2* gene can be eliminated without affecting cell growth on either rich or minimal growth medium (33). The *Drosophila* gene *knirps* encoding a key transcriptional regulator in embryonic development can be eliminated without affecting head development, during which *knirps* is expressed (197). And finally, there are the three *TPK* genes in yeast, which encode catalytic subunits of the yeast cyclic AMP-dependent protein kinase, a key molecule in cell signaling. Any two of these three genes are dispensable for cell growth (553). The last example contains a hint that redundancy may be responsible for genes whose deletion has no effect: The three *TPK* genes form a family of genes that originated from two duplications of a single ancestral gene (553). Closer inspection of the previous examples also reveals redundant duplicate genes: Like the *TPK* genes, the *CLN1* gene has two duplicates, *CLN2* and *CLN3*, with similar functions; the *BarH2* gene has a duplicate, *BarH1*; the *HMG2* gene has a duplicate *HMG1*; and *knirps* has a duplicate, *knirps-related* (33, 197, 230, 504). Eliminating *all* duplicates of a gene often reveals the severe phenotypic effects that mutations in the gene lack, as expected if redundancy is the cause for weak gene knock-out effects. Redundant duplicate genes with similar functions can thus help explain why knock-out mutations in important genes have no phenotypic effects.

Gene duplications are abundant by-products of recombination and DNA repair processes in most genomes. Typically, between 25 and 50% of eukaryotic genes have at least one duplicate within the same genome. Immediately after a gene duplication, two functional duplicate genes often produce the same two polypeptides with the same function. Subsequently, mutations that accumulate in either gene can lead to elimination of one duplicate, or to divergence in the duplicate's functions (553). Although such divergence is often very limited, as the examples above show, most gene duplicates do not have completely identical functions. That is, they often encode multifunctional proteins that share some functions while they differ in others. For example, the *knirps* gene is dispensable for head development, but it is necessary for abdominal development (197). Mig1p and Mig2p, two yeast transcriptional regulators, act redundantly in repressing the yeast gene *SUC2*, needed for metabolizing sucrose, but Mig1p is uniquely required for repressing the *GAL* genes necessary for galactose metabolism (346). Similarly, the three *TPK* genes, any two of which are normally dispensable for cell division, have different functions under conditions where yeast forms filamentous cell aggregates called pseudohyphae. *TPK2* is an essential gene under these conditions (464). Such observations have led to the notion that duplicate genes can have partially redundant or overlapping functions.

I note in passing that key concepts of chapters 13 and 14 on neutral spaces and evolvability apply also to gene redundancy. Take first the concept of a neutral space. Each gene in a family of  $n$  fully redundant genes—originated through multiple gene duplications—can suffer mutations that may alter or eliminate the gene's function. In a simple neutral space representation, we might ascribe a value of 1 to a gene whose function has remained intact and identical since the duplication, and a value of 0 if its function has been altered or eliminated. We can then think of the whole gene family as a string of zeroes or ones, or as a point in an  $n$ -dimensional discrete space. Only a number  $k < n$  (minimally,  $k = 1$ ) of these genes may have to be intact to perform a task critical to the organism. If so, then all the points corresponding to strings with more than  $k$  entries of 1 form a neutral space. This concept extends easily to more sophisticated notions of gene function, allowing for multifunctional and partially redundant genes.

Gene redundancy is similarly linked to evolvability, the central topic of chapter 14. There, I argued that neutral genetic change—whose incidence increases with increasing robustness—does not hinder but promotes evolutionary innovation. Gene redundancy provides an excellent example supporting this notion. As long as one copy of a redundant gene retains an essential function, mutations in the other copy have a smaller likelihood of being deleterious. This results in more mutations being neutral,

which facilitates the evolution of new gene properties, properties that can eventually turn into adaptations. Multiple examples of gene duplications followed by functional innovation exist. They include the evolution of butterfly photoreceptor genes with sensitivity to an increased fraction of the visible electromagnetic spectrum, and the diversification of the development and anatomy of vertebrates, for which extensive duplication of developmental genes may have played an important role (63, 241, 643).

### The Value of Genome-Scale Gene-Deletion Studies

Examples, however many, of individual redundant genes, can provide no more than anecdotal evidence for the prevalence of gene redundancy. Ideally, one would like to find out how prevalent gene redundancy is on a genome-wide scale. To do so, one needs to carry out deletion studies that eliminate many genes in a genome and systematically assess the effect of these deletions. The first such large-scale study created some 300 different strains of budding yeast, each of which lacked one of the genes on chromosome V (504). This study was followed by larger scale studies in yeast, the worm *Caenorhabditis elegans*, and the plant *Arabidopsis thaliana* (15, 184, 268), studies that mutated the DNA or reduced the expression levels of thousands of genes. A caveat to comparing the results of such studies is that their experimental approaches differ. For instance, although deletion of specific genes through homologous recombination is highly effective in yeast, it is not feasible on a large scale in higher organisms such as *C. elegans*. In such organisms temporary gene activation through interference with the expression of messenger RNA is the method of choice (268).

The first surprise of systematic gene knock-out studies is the sheer number of genes with no detectable effect. For instance, the systematic knock-out of most chromosome V genes in yeast shows that almost 40% of genes have no detectable effect on key fitness indicators such as cell division rate and sporulation efficiency in five different nutritional environments (504). Later studies that eliminated more than 90% of all yeast genes yielded similar results (184, 615).

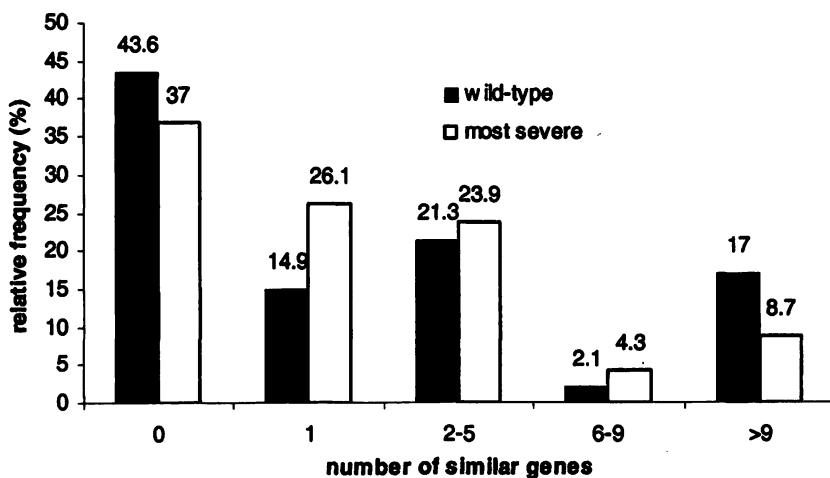
Such striking numbers raise the question whether genes with no apparent effect are truly dispensable under all circumstances. This question brings me back to the discussion of neutrality in chapter 14. Are null mutations in such genes neutral in the traditional, essentialist sense, or are they neutral in a more limited sense? As I argued in chapter 14, no single laboratory experiment could answer this question definitively. However, the likely answer is that such mutations are neutral in the more limited sense I discussed in chapter 14. First, it is easy to conceive of genes with specific functions, such as metabolic genes, where a mutation may have

no effect in one environment but may be fatal in another environment. For metabolic genes, such environmental specificity may be the rule rather than the exception (247). Even very rare environmental conditions, conditions that may occur only once every few thousand generations, can cause such genes to be maintained in a population (588). Second, if genes whose elimination has no effects in one or a few environments were truly dispensable, they should evolve much more rapidly than indispensable genes, because they would be able to tolerate more mutations. However, the statistical association between a gene's dispensability and its rate of evolution is weak at best (233, 234, 262, 427, 637). Unfortunately, except for the limited number of metabolic genes (fewer than 20% of genes in a genome), it can be very difficult to determine directly whether a gene is not used in a particular environment or physiological condition, especially in higher eukaryotes, where “gratuituous” expression of genes may be frequent.

### Genome-Scale Gene-Deletion Studies and Redundancy

Even though their neutrality may be only temporal, the sheer number of genes—thousands—with no deletion effect still needs a mechanistic explanation. Does gene redundancy provide this explanation? Given what I have said so far, one would think that the answer should be yes. First, there are many anecdotal examples of redundant genes. And, second, up to 50% of genes in eukaryotic genomes have duplicates (85, 451, 469). However, the results of genome-scale gene-deletion studies reveal the next surprise: Thousands of genes whose deletion has no detectable effect are single-copy genes. They have no duplicates in the genome.

The results of an early pertinent study are summarized in Figure 15.1 (584). In this figure, I grouped gene-deletion mutations of yeast chromosome V into two categories: those with a nondetectable defect, and those with a severe defect leading to a growth rate reduction of more than 25% relative to the wild-type. The figure's vertical axis shows the proportion of genes in each defect category that have the number of duplicates indicated on the horizontal axis. Significantly, more than 40% of duplicate genes with weak or no fitness effect are single-copy genes. A more recent study by Gu and collaborators that used more than 4700 growth rate measurements of yeast gene-deletion strains reached a qualitatively identical result: 39.6% of yeast genes with a weak or no fitness defect have no duplicate (216). Additionally, even if a gene has a duplicate, the duplicate may have diverged in function and may not be responsible for a weak gene-deletion effect. The situation in many-celled eukaryotes might be fundamentally different, because they contain more duplicates which



**Figure 15.1** Many genes with no detectable effect on fitness indicators are single-copy genes. All genes on yeast chromosome V are divided into two categories, those with the most severe fitness effects when eliminated from the genome (white bars), and those with a phenotype indistinguishable from wild-type (black bars). Numbers shown above bars are percentages of genes on chromosome V with the number of duplicate genes in the yeast genome shown on the horizontal axis. From Figure 5 in (584).

occur in larger families (85, 451, 469). However, a recent study using the results of RNA interference experiments that temporarily silenced more than 16,000 worm genes suggests otherwise. In this study, Gavin Conant and I found that more than 7500 single-copy genes show no detectable phenotypic effect when temporarily silenced, versus 4515 of genes with one or more duplicate (86).

Does all this mean that gene redundancy is unimportant in determining the effect of gene deletions? No. For genes with duplicates, the likelihood that a gene has a lethal effect is lower than for single-copy genes (86, 216). Moreover, the proportion of genes with weak or no effects is higher for duplicate genes than for single-copy genes (86, 216). Third, the more similar two duplicates are, the less severe is the effect of deleting or silencing one of them (86, 216). Fourth, the larger the number of duplicates a yeast gene has, the faster the gene evolves, implying that it can tolerate more mutations (85). And finally, pairs of duplicate genes have more similar phenotypic effects after deletion than randomly chosen pairs of unrelated genes (216). Thus, gene duplications clearly play a role in determining gene-deletion effects. However, the above numbers also show that gene redundancy is clearly not responsible for the overwhelming majority of weak gene-deletion effects.

## Rapid Functional Divergence of Duplicate Genes

The above results suggest that both distributed robustness and gene redundancy play a role in mutational robustness. However, they leave a more quantitative question unanswered: On a genome-wide scale (and not just for one functional class of genes) what proportion of weak gene perturbation effects are due to gene duplication? An estimate of this proportion based on large-scale gene deletion experiments suggests that in yeast between 23 and 59% of weak gene perturbation effects are due to gene duplication (216). A more recent computational analysis in yeast indicates a greater role for gene duplicates (247). However, this study is restricted to metabolic genes—only some 16% of all yeast genes—and it takes environmental specificity in a limited number of environments into account, which makes its results difficult to compare with these figures. In a higher organism, the worm *C. elegans*, the proportion of weak gene perturbation effects due to gene duplication may be lower than in yeast, between 3 and 36% (86).

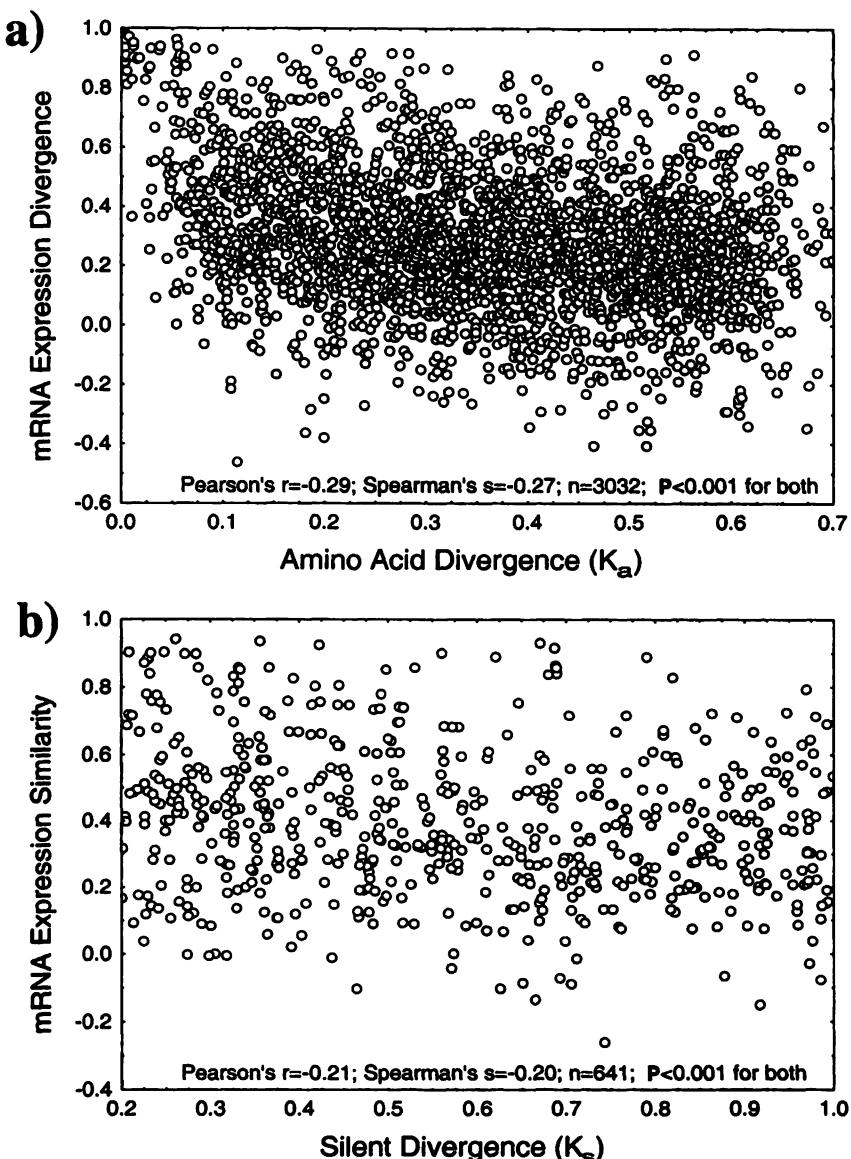
Such results make it seem unlikely that gene redundancy is responsible for the vast majority of weak gene perturbation effects, although the numbers in the worm have to be taken with a grain of salt, because they are based on results of RNA interference assays. The true percentage of weak gene deletion effects would be close to the upper bound of the above intervals (59% for yeast) if most duplicates retained similar functions long after their duplication. It would be close to the lower bound (23%) if most gene duplicates diverged rapidly in function. In the following section, I will argue that many duplicate genes diverge rapidly in important indicators of gene function. This suggests that the true proportion of weak gene deletion effects due to gene duplication is nowhere near the upper bound.

Estimating the rate at which gene duplicates diverge in function faces two technical problems. The first of them is to measure the time that elapsed since a gene duplication. The crudest indicator of this time is the number of nucleotide changes that lead to amino acid changes in either of the duplicates' gene product. This number is roughly proportional to the time since duplication. The problem with this indicator of time is that some amino acid changes are favored by natural selection and can thus accumulate much more rapidly than changes that do not affect protein function at any one time. This can lead to large errors in divergence time estimates. The problem can be alleviated by estimating instead the number of synonymous (silent) nucleotide substitutions that occurred in two genes since the duplication. Silent substitutions are substitutions that do not lead to amino acid changes in an encoded protein. They are subject

to much weaker selection than amino acid replacement substitutions, and thus do not pose the same problem as amino acid substitutions (336). In organisms where fossils can be used to calibrate this molecular clock, silent divergence between genes has even been mapped onto absolute (geological) time. A note of caution, however, is that selection can affect silent nucleotide changes to some extent, such that silent divergence rates can also vary among gene duplicates and even among parts of a gene. In practice, amino acid divergence is commonly estimated through the fraction  $K_a$  of nucleotide substitutions that occurred since the duplication at non-synonymous nucleotide positions. The synonymous (silent) divergence of two duplicate genes is measured as the fraction  $K_s$  of silent substitutions that occurred since the duplication at synonymous nucleotide positions (336). These two indicators of amino acid and silent divergence take into account the different lengths of different genes.

The second problem with this approach is to identify whether two gene functions are identical, similar, or completely different. To identify one gene's function—let alone two—is not trivial, as my discussion of multi-functional proteins in chapter 14 showed. Yes, even how to properly define gene function is problematic, because gene function can mean many different things. For instance, gene function can be defined in biochemical terms, such as through a gene product's DNA binding and catalytic activity, or via biological processes, such as through a gene's role in liver metabolism or brain development (27). To make matters worse, assessing whether gene duplicates *generally* retain similar functions can be answered only by characterizing hundreds or thousands of gene duplicates. The only current solution to this problem is to use crude indicators of gene function, indicators that are measurable for many genes. Functional genomics provides a wealth of such indicators, including where and when a gene is expressed, which proteins regulate its transcription, and which other molecules its protein products interact with. Each such indicator captures only one dimension of gene function, but it has the advantage that it can be measured for thousands of genes at once.

Do most duplicate genes retain similar expression patterns, molecular interaction partners, or common transcriptional regulation long after duplication? No. Even many recently duplicated genes show highly different mRNA gene expression patterns, so much so that there is at best a weak correlation between the silent divergence of two duplicate genes and the similarity in their expression patterns (215, 583). Figure 15.2 shows an example from the nematode worm *C. elegans* (86). The figure is based on an analysis of more than 500 different microarray gene expression measurements and sequence information from the whole worm genome. It shows that many gene duplicates that produce highly similar proteins (Figure 15.2a) or that have arisen recently (Figure 15.2b) show



**Figure 15.2** Duplicate genes diverge rapidly in their mRNA expression pattern. mRNA gene expression similarity (vertical axes) of duplicate genes in the worm *C. elegans*. The amino acid divergence  $K_a$  and synonymous divergence  $K_s$  of each duplicate gene pair are shown on the horizontal axes of (a) and (b), respectively. mRNA gene expression similarity is measured by the Pearson product-moment correlation coefficient of logarithmically transformed gene expression ratios from 522 microarray experiments (283). The nonsynonymous (amino acid replacement)

very different mRNA expression patterns, almost as different as genes that have duplicated a long time ago.

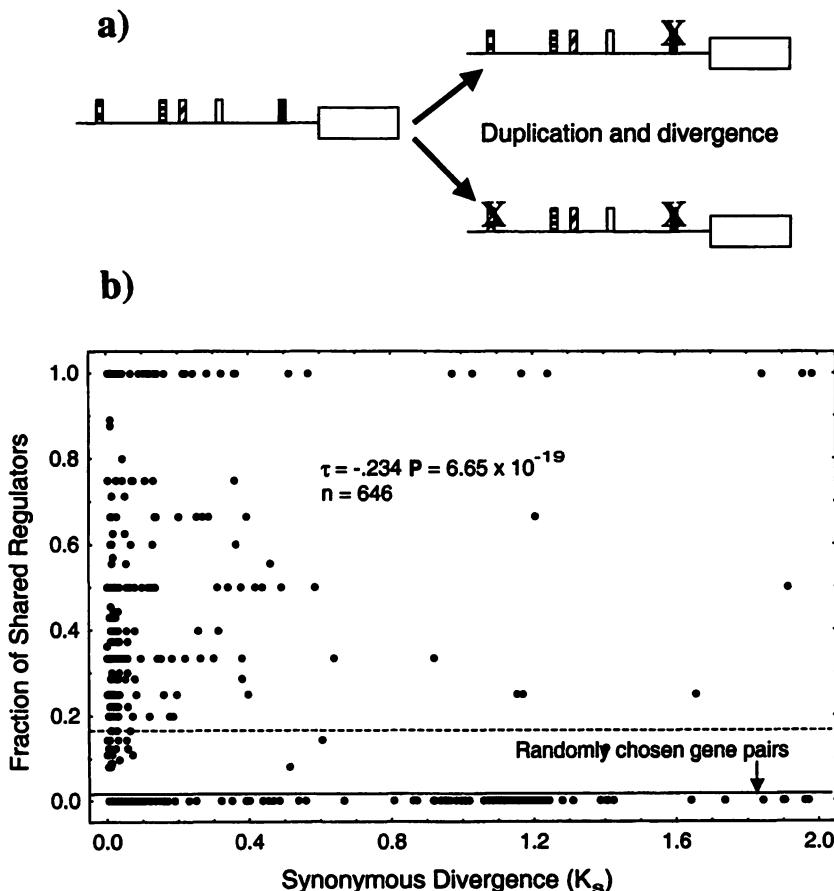
A similar pattern holds for the transcriptional regulators that bind at regulatory regions of duplicate genes (143). After a gene duplication, the regulatory regions of two duplicate genes diverge. Much of the initial divergence occurs through the disappearance of binding sites for different transcription factors, because random mutations are more likely to eliminate such binding sites than to create new ones. Binding of transcriptional regulators to a large fraction of yeast genes has been studied using the technique of chromatin immunoprecipitation (325). Figure 15.3 shows how the similarity in the spectrum of transcriptional regulators that bind to the regulatory region of two duplicate genes depends on the silent (synonymous) divergence between the duplicates (143). Such divergence is a crude indicator of the time since duplication. For yeast, a synonymous divergence of  $K_s = 0.1$  among duplicated genes corresponds to a duplication age of approximately 10 million years (586, 587). The solid horizontal line indicates the average fraction of shared transcriptional regulators between pairs of genes chosen at random from the yeast genome. The figure demonstrates that the regulatory regions of many closely related duplicate genes are bound by completely different transcriptional regulators. This is further indication that functional divergence among duplicate genes is often rapid.

Finally, genes whose products interact with other proteins show a rapid divergence of common protein interactions (586, 587). An example of such rapid divergence is shown for duplicate yeast genes in Figure 15.4. Even among the most recent gene duplicates, with a silent divergence of  $K_s < 0.2$ , fewer than 70% of gene products share a protein interaction partner. Among older duplicates, fewer than 20% have any common protein interaction partners. Qualitatively, these findings are independent of which among various genome-scale datasets on protein interactions (181, 253, 372, 561) one uses in this analysis.

Each genome-scale data set on indicators of gene function has its own source of noise, but taken together, the above evidence suggests that

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divergence of two duplicate genes is measured by the fraction  $K_a$  of nucleotide substitutions that occurred since the duplication at nonsynonymous nucleotide positions. The synonymous (silent) divergence of two duplicate genes is measured as the fraction  $K_s$  of synonymous substitutions that occurred since the duplication at synonymous nucleotide positions. Synonymous substitutions are nucleotide substitutions that did not lead to amino acid replacements. Panel (b) does not include gene duplicates with  $K_s < 0.2$ , because microarray experiments cannot distinguish differences in expression among such closely related duplicates. Data from (86).



**Figure 15.3** Duplicate genes diverge rapidly in the binding of transcriptional regulators to their regulatory regions. (a) After a gene duplication, the regulatory regions of two duplicate genes diverge. Much of this divergence occurs through the disappearance of binding by individual transcription factors (indicated by the small rectangular boxes), because random mutations are more likely to eliminate transcription factor binding sites than to create new ones. (b) The vertical axis shows the fraction  $f$  of transcriptional regulators that bind to the regulatory region of *both* genes of a pair of yeast gene duplicates whose synonymous divergence is shown on the horizontal axis. If  $d_1$  transcriptional regulators bind to the regulatory region of gene 1, if  $d_2$  transcriptional regulators bind to the regulatory region of gene 2, and if  $d_{12}$  transcriptional regulators bind to the regulatory regions of both genes, then this fraction  $f$  calculates as  $f=d_{12}/(d_1+d_2-d_{12})$ . A value of  $f=1$  indicates that the same transcriptional regulators bind the two genes' regulatory regions. A value of  $f=0$  indicates that completely different regulators bind the two genes' regulatory regions. The horizontal lines indicate the average (solid) plus one standard deviation (dashed) fraction  $f$  of shared transcriptional

duplicate genes do not, as a rule, retain similar functions long after the duplication. It provides further evidence that redundancy is not the dominant factor determining robustness to mutations. Again, this does not mean that genes never retain similar functions long after duplication. For instance, some of the gene families listed above, families whose members retained similar functions, result from very ancient gene duplications. Take the CLN family of partially redundant cell-cycle regulators. The most closely related genes in this family are CLN1 and CLN2. Since their duplication, more than  $K_s = 1$  synonymous nucleotide substitutions have occurred per synonymous site on their DNA. By this measure, this duplication may be over 100 million years old. Another example involves the three TPK genes from yeast, any two of which are dispensable for growth. Here, the most closely related pair is TPK1-TPK3, also with  $K_s > 1$ . We do not know why some gene duplicates retain similar functions long after others have completely diverged in function. However, part of the answer may be that selection for robustness can delay or stop the functional divergence of some genes, a possibility I discuss further in chapter 16.

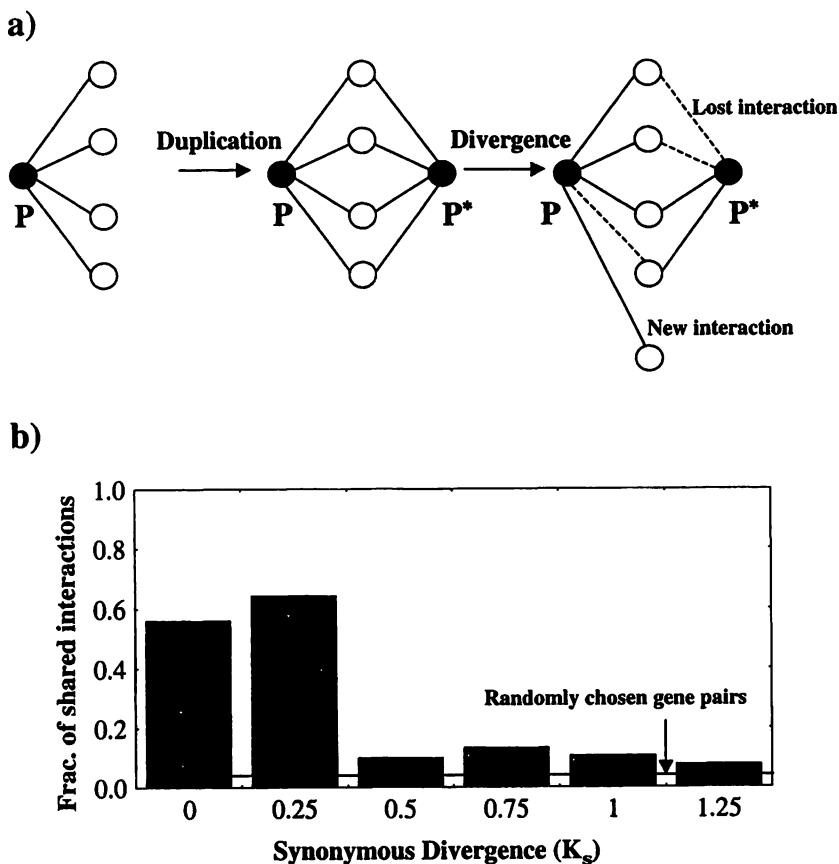
### Distributed Robustness

If gene redundancy is not the major cause of many gene deletion's small effects, then what is? The answer is a phenomenon one might call distributed robustness. In distributed robustness, many parts of a system contribute to its function, but all of these parts have different roles. When one part fails or is changed through mutations, other parts can compensate for this failure, but not simply by standing in for the failed part. Most well-studied examples of robust systems—examples I discussed in parts I and II—are examples of distributed robustness. This is no coincidence. It is a reflection of the prevalence of distributed robustness over simple redundancy of parts in biological systems.

Among the examples I discussed earlier, the most straightforward comparison to robustness through gene redundancy is provided by the flux-balance analysis of metabolic networks (chapter 9). There, a kind of genetic change analogous to gene deletions—the complete elimination

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regulators between pairs of genes chosen at random from the yeast genome. The synonymous (silent) divergence of two duplicate genes is measured by the fraction  $K_s$  of synonymous substitutions that occurred since the duplication at synonymous nucleotide positions (586, 587). From (143), which uses transcription factor binding data based on chromatin immunoprecipitation (325).



**Figure 15.4** Duplicate genes whose products interact with other proteins diverge rapidly in their common interactions. (a) Shortly after a gene duplication, the products  $P$  and  $P^*$  of two duplicate genes interact with the same proteins. Eventually, some or all of the common interactions will be lost, and new interactions may be gained by either protein. The number of common interaction partners can be used as a crude indicator of functional similarity among proteins. (b) The horizontal axis corresponds to duplicate gene pairs in the protein interaction network of the yeast *Saccharomyces cerevisiae*, binned according to their evolutionary distance, as measured by the fraction of synonymous (silent) substitutions at synonymous sites,  $K_s$  (336). The vertical axis shows the proportion of gene pairs in each bin whose products have *at least* one interaction partner in common. The horizontal line indicates the fraction of protein pairs chosen at random from the yeast genome that have *at least* one common interaction partner. Protein interaction data are taken from high-throughput genome-scale screens of protein interactions (253, 561) and from a public database of such interactions (MIPS), as described in (372). Similar results (not shown) are obtained when these data sets are considered separately. Qualitatively similar results are also obtained when experimental data of protein complex composition (181) are used in such an analysis.

of metabolic reactions—is well-studied. Note that these metabolic networks do not contain redundant reactions. That is, each and every chemical reaction is different. Yet over 50% of these reactions, some even in central parts of metabolism, can be eliminated without affecting metabolic output in one environment. Why? Because the network is able to reroute metabolic flux through parts unaffected by the eliminated reactions. Characteristically for distributed robustness, such compensatory changes can take place in parts of the system far removed from the mutated part. A case in point is the first reaction of the pentose phosphate shunt discussed in chapter 9. Its elimination affects growth minimally. Despite this small effect on growth, the elimination of this reaction results in large compensatory flux changes elsewhere in the network. Some of the largest such changes occur in glycolysis, as well as in the mode of NADPH biosynthesis: Before the mutation, much NADPH is produced in the pentose phosphate shunt, but afterward NADPH is largely produced from NADH through the transhydrogenase reaction, which is not part of the shunt. Such compensatory changes, which occur in parts of metabolism superficially far removed from the affected reaction, are necessary to keep the effects of the reaction elimination on the network to a minimum.

Other example systems from part I and II cannot be directly compared to systems whose parts are genes and whose robustness is to gene deletions. Either the system exists on a different levels of biological organization, where the system's parts are not genes but nucleotides, amino acids, or whole cells; or the system's robustness to gene deletions is unknown, and only other kinds of mutations, such as those affecting regulatory interactions among genes, have been studied. Despite such incommensurability, it does not take much effort to see that redundancy of parts—properly defined—is similarly not the predominant cause of the robustness of these systems.

A case in point are genetic regulatory networks like the segment polarity network and, more generally, regulatory networks with cooperative interactions (chapter 10). No two genes in such networks need have the same function, yet the networks can be highly robust to changes in regulatory gene interactions. Another example comes from the metabolic control models of enzymatic pathways (chapter 8). Here, a substantial change in an enzyme's activity may have negligible effects on the flux of matter through the pathway. However, as a rule, any two enzymes in the pathway catalyze completely different chemical reactions. Again, robustness emerges not from redundancy but from the cooperation of enzymes with different activities.

On higher levels of biological organization, we know less about individual genes involved in a system's performance, but one can infer indirectly that redundancy is not central to robustness. For instance, the

genes responsible for the embryonic development of many phenotypic characters harbor much variation that never affects the phenotype. Are redundant gene functions responsible for such cryptic variation? Probably not. If they were, then mutations that reduce this robustness would preferably affect genes with redundant duplicates. However, the mutations that reduce robustness most drastically are completely different in nature (chapter 11). Take the nonredundant gene encoding the heat-shock protein Hsp90, for which heterozygous loss-of-function mutations abolish robustness in a wide spectrum of developmental pathways. The reason is that Hsp90 interacts with more than 80 different proteins important in development, and helps stabilize their conformation.

On the highest, whole-organism level, we can ask how adult parasitic wasps or sea urchins can remain unchanged after a complete reorganization of embryonic development within a few million years. Is it redundancy of parts, perhaps of cells within the embryo, that are produced in excess and can be used for new purposes that allows such a reorganization to go unpunished? The realization that the fates of each cell, even in an early 32-cell sea urchin embryo, can be radically transformed (Figure 12.7) renders this suggestion an unlikely possibility.

On the lowest levels of biological organization, redundancy similarly does not suffice to explain robustness. Here, the paradigmatic examples are biological macromolecules, such as proteins and RNA, because any living cells contains countless millions of them. Their folding in space involves the cooperation of hundreds of building blocks—nucleotides or amino acids—each of which forms unique (nonredundant) interactions with other building blocks. If a change in one amino acid leaves a protein's three-dimensional conformation unchanged, is this because the protein contained a redundant “backup” amino acid? Clearly not. The thermodynamic stability of any macromolecular structure is the sum of all contributions that such interactions make to the structure's free energy.

But what about the genetic code? Isn't its redundancy at the heart of its robustness to nucleotide changes? Yes and no. Codons in the genetic code are redundant, because two codons can encode the same amino acid. Such redundancy is an inevitable consequence of any genetic code that uses 61 triplets of nonoverlapping nucleotides to encode 20 amino acids. The universal genetic code, however, has evolved robustness far above that of the average triplet-based code (chapter 3), despite the fact that the universal code and the “average” code are redundant to the same degree. This increased robustness is a result of exactly how the universal code maps the 64 possible codons onto 20 amino acids. It is much less clear whether to call this “excess” robustness a result of redundancy.

## Quantifying Redundancy and Distributed Robustness?

In sum, multiple examples from previous chapters emphasize the role of distributed robustness in biological systems. In some of these examples, such as proteins that fold into a three-dimensional structure, the question whether two parts are redundant has little meaning. In other systems, such as genetic and metabolic networks, redundancy of parts plays some role in explaining robustness to mutations. However, even though minor, the influence of redundancy on these systems need not be negligible. Can we quantify the extent of redundancy in a genetic system? On a crude level, this seems quite feasible. For example, metabolic reactions in living cells are driven by enzymes, some of which may be the products of duplicate genes. One could simply determine the fraction of enzymes in a metabolic pathway that are encoded by more than one gene and use this fraction as an indicator of redundancy in the network. However, can one go beyond that? Specifically, for any specific indicator of a system's performance, can we say to what extent its robustness is caused by redundancy or by distributed robustness?

A first observation is that the answer to this question must depend on how one defines robustness. For instance, a flux-balance representation of metabolic networks, and a differential equation representation of a regulatory gene network are fundamentally different, and will therefore also employ different measures of robustness. But more disturbingly, redundancy and distributed robustness can have indistinguishable effects on a system. I use a simple example from a metabolic system to make this point.

In chapter 8, I discussed how we can understand the robustness of metabolic pathways like that shown in Figure 15.5a to changes in enzyme activities. An important concept in this regard is that of an enzyme's flux-control coefficient. This coefficient indicates by how much a change in an enzyme's activity changes the rate at which some substrate is converted into an end product of the pathway. The most convenient mathematical representation of this coefficient is a differential quotient, which measures the response of flux to a very small change in enzyme activity. Consider, for simplicity, a pathway like that in Figure 15.5a, where every enzyme  $E_j$  ( $1 \leq j \leq n$ ) has the same activity  $e$ . Using equation 8.4, the flux-control coefficient of each enzyme calculates as

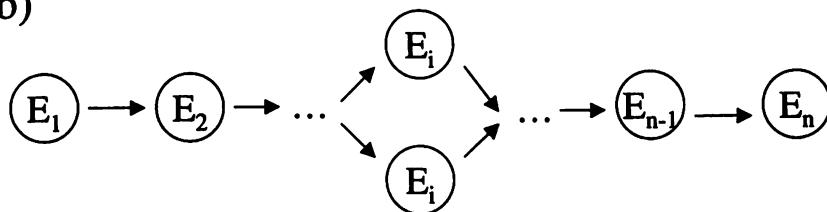
$$C_j = \frac{\partial F}{\partial E_j} \frac{E_j}{F} = \frac{1/E_j}{1/E_1 + 1/E_2 + 1/E_3 + \dots + 1/E_n} = \frac{1/e}{1/e + \dots + 1/e} = \frac{1}{n}$$

$1 \leq j \leq n \quad (15.1)$

a)



b)



**Figure 15.5** (a) An enzymatic pathway consisting of  $n$  enzymatic reactions catalyzed by enzymes  $E_1$  through  $E_n$ . (b) The same pathway, but now the gene encoding enzyme  $E_i$  has been duplicated, introducing redundancy into the pathway.

This pathway is robust to mutations in the sense that a small reduction in the activity of any one enzyme activity changes the steady-state pathway flux by an amount that is a factor  $n$  smaller. The more enzymatic steps the pathway comprises, the higher is the pathway's robustness to a given reduction in one enzyme's activity. Most importantly, this robustness is evenly distributed. That is, each and every enzyme  $E_i$  has the same effect on flux, the same flux-control coefficient. Even distribution of flux control among system parts may seem a reasonable way to make the concept of distributed robustness exact.

With this simple observation as a starting point, imagine that we introduce redundancy into this pathway, as shown in Figure 15.5b. Specifically, we duplicate the gene encoding one of the enzymes, say enzyme  $E_i$ . Two copies of the gene produce twice the amount of enzyme, and as a result, this enzyme's activity, as defined in chapter 8, doubles to  $2e$  (528, chapter 8). (In engineering, this kind of redundancy would be called parallel and active redundancy, because both copies of the system part are active—expressed—and their products act in parallel (177, Chapter 6).) How does this duplication affect robustness of the pathway? For enzyme  $E_i$ , the control coefficient now calculates as

$$C_i = \frac{\partial F}{\partial E_i} \frac{E_i}{F} = \frac{1/2e}{1/2e + \sum_{k=1}^{n-1} 1/e} = \frac{1}{2n-1} \quad (15.2)$$

If the number  $n$  of enzymes  $n$  in the pathways is large, then the control coefficient of this enzyme has been effectively cut in half. In other words, the pathway is now nearly twice as robust to changes in activities of this enzyme. This is the main effect of introducing two copies of its gene. What about the remaining enzymes, whose genes have not been duplicated? Their control coefficient calculates as

$$C_j = \frac{\partial F}{\partial E_i} \frac{E_j}{F} = \frac{1/e}{1/2e + \sum_{k=1}^{n-1} 1/e} = \frac{2}{2n-1} \quad j \neq i \quad (15.3)$$

It has slightly increased from the previous value of  $(1/n)$ . If the number of enzymes  $n$  is large, however, then this control coefficient is almost unchanged. If we were to introduce not one but  $k > 1$  additional copies of the gene for  $E_i$ , then the enzyme's control coefficient would further decrease and that of the other enzymes would increase slightly. The pathway would become increasingly more robust to changes in the activity of  $E_i$ . In sum, the net effect of introducing gene redundancy into this metabolic pathway is to render the previously uniform distribution of control coefficients (15.1) nonuniform. From this perspective, one might propose to distinguish redundancy from distributed robustness through an uneven distribution of control in the pathway. Redundancy causes uneven distribution of flux control across parts of the pathway. The lower an enzyme's control coefficient, the higher its redundancy.

It does not take much thinking to discover the fundamental flaw in this argument. Uneven distribution of control can be achieved by means that have nothing to do with redundancy. For example, even if each enzyme is encoded by only one gene, and even if each enzyme-coding gene expresses the same numbers of enzyme molecules, each enzyme molecule could have different catalytic activity, which would cause an uneven distribution of flux control. Similarly, if we duplicated all genes in the model pathway of Figure 15.5a, every enzyme in the pathway would have the same flux-control coefficient—flux control would be perfectly distributed—but now all enzymes are encoded by two redundant genes. In sum, a pathway could have a uniform distribution of control coefficients with much gene redundancy, or a nonuniform distribution without any gene redundancy.

All this implies that our ability to determine how much redundancy a system contains depends on our perspective. If we view a metabolic pathway from the point of view of the genes that encode its enzymes, it may be simple to determine how much redundancy it contains—just count the redundant genes. The same holds if we view the pathway from the perspective of the individual protein molecules that do the chemical

work: It is easy in principle to estimate how many of the molecules catalyzing the same reaction a cell contains, and all of these can be viewed as redundant. However, as I just showed, assessing the extent of redundancy becomes impossible on the level of enzyme activities, the aggregate variables best suited to understand a pathway's robustness.

This problem is very general and not a peculiarity of metabolic pathways. For instance, quantities suitable to measuring the robustness of gene regulatory networks have been defined, quantities that are exactly analogous to metabolic control coefficients (480, 481). One can characterize the robustness of any property of such a network—the expression level of its genes, their products' activities or phosphorylation state, or their affinity to other molecules—by such control coefficients. And for any network design devoid of redundant genes, there may be a design including redundant genes that shows exactly the same distribution of control coefficients. Even more generally, analogous insights exist in control theory, a discipline aiming at understanding and controlling the behavior of any system, whether natural or engineered. An important concept in control theory is that of the transfer function, which specifies how a system's output behaves as a function of its input. General theorems of control theory imply that for any system with redundant parts, one can design a system without redundancy but with the same transfer function, i.e., the same behavior (327, chapter 4).

All this implies that the qualitative approach to estimating the extent of redundancy in a biological system—enumerating redundant parts—may be the only one. Beyond such enumeration, it may be impossible to distinguish distributed robustness and redundancy, because the two have indistinguishable signatures on the variables necessary to understand a system's function and robustness.

To summarize, redundancy as a source of robustness is especially prominent for systems whose parts are genes and gene products. Its importance results from the abundance of gene duplications in most genomes. In such systems, gene redundancy is partly responsible for increased robustness to mutations and an increased incidence of neutral mutations. This increased neutrality is important for evolutionary innovation, the evolution of new gene functions. However, redundancy is not the most prominent cause of robustness on this level of biological organization. This is because typically fewer than half of a genome's genes are duplicates, and many of these duplicates diverge rapidly in function after duplication. In addition, many genes whose elimination has no effect on the organism are genes without duplicates. A comparison of biological systems on multiple levels of organization corroborates this notion: Not redundancy of parts but distributed robustness is the major cause of robustness in living systems.

# 16

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## Robustness as an Evolved Adaptation to Mutations

This chapter focuses on the question of how natural selection can increase a biological system's robustness to mutations. As I argued earlier (chapter 13), robustness to mutations can have two major ultimate causes. First, it may be a by-product of how evolution searches for solutions to problems that organisms face. Second, in a system that embodies any one such solution, robustness can further vary and increase in evolution, as examples scattered through earlier chapters showed. Specifically, we saw earlier that robustness in systems as different as macromolecules and genetic networks *can* increase in evolution. In addition, in systems like the genetic code and developmental pathways, there is evidence that it *did* evolve.

When speaking of a system's evolved mutational robustness, I mean robustness that has increased in evolution because it provides a benefit in and by itself, robustness that is an adaptation. Mutational robustness may be an adaptation, but an adaptation to what? This and the following chapter address this question. Specifically, this chapter examines the obvious answer: Mutational robustness is an adaptation to mutations. The next chapter deals with an equally if not more important answer: Robustness to mutations can be an adaptation to environmental change and molecular noise inside the organism. In other words, robustness can be an adaptation to nongenetic change.

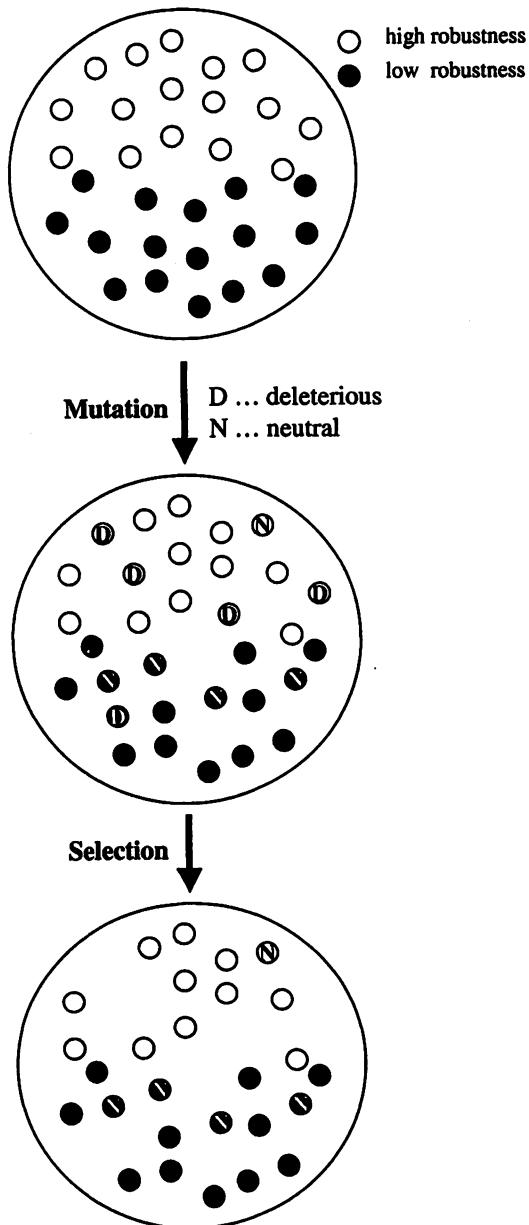
Importantly, as we will see, both of these explanations for the evolution of robustness require selection only on the level of the individual, not on the level of a group or species. To be sure, if robustness is advantageous to the individual, it may also be advantageous to groups or species. However, group selection may not occur in all organisms, and its incidence is still controversial (413, 507). In contrast, all organisms are subject to selection among individuals. Individual selection is thus a more robust explanation—pardon the pun—for evolved robustness. A possible exception to this prevalence of individual selection holds for cells inside many-celled organisms. Such cells face mutations that may lead them to divide uncontrollably and thus endanger the well-being of the whole. It is

possible that policing mechanisms, such as programmed cell-death of cancerous cells, have evolved explicitly to rid the organism of such renegade cells (306, 308). Such mechanisms could endow the whole organism with robustness to particular classes of mutations. Individual cells would remain sensitive to such mechanisms, however, not because of any advantage they derive from such sensitivity. To the contrary, to any one cell such mechanisms can spell death. Susceptibility to policing mechanisms can thus not evolve or be maintained through natural selection on the level of individual cells, but only because it benefits an entire cell lineage or the whole organism.

Multiple studies in population genetics and quantitative genetics ask how robustness evolves (55, 141, 228, 274, 305, 404, 415, 448, 462, 499, 562, 579, 582, 585, 591, 592, 610). To compare their results in detail would be a daunting task, because most of these studies are built on mathematical models of evolution, and subtly different assumptions in such models can yield drastically different results. Instead, I will discuss two simple yet general principles that emerge from most models. Then, I will present two such models in greater detail, to show the manifestations of these general principles, and to highlight differences among them. These differences suggest the futility of searching for a universal theory to explain robustness as an adaptation to mutations for all conceivable biological systems.

### **Selection for Robustness Is Indirect, “Second-Order” Selection**

The evolutionary mechanism generating robustness as an adaptation to mutations is highly unusual. Evolution by natural selection acts through the differential survival and reproduction of organisms. But do two biological systems identical in everything but their mutational robustness have different abilities to survive and reproduce? No. (I will speak of systems in the most general terms here, because the principle applies on all levels of organization, but it may be helpful to think of two proteins differently robust to amino acid changes, or two gene networks differently robust to changes in regulatory gene interactions.) Two systems that differ in robustness may differ in only one respect: A random genetic change in the more robust system is less likely to impair its function. Thus, until they suffer mutations, these systems may be indistinguishable. Put differently, their genotypic differences may be phenotypically neutral. However, once affected by a random mutation, the more robust system will allow its carrier to continue to survive and reproduce, whereas the less robust system may fail. Figure 16.1 shows a visual caricature of this process. Over many



**Figure 16.1** Natural selection increases robustness indirectly, through mutations that affect robust systems to a lesser degree than fragile systems. The large circle symbolizes a population; the smaller circles symbolize organisms in the population, organisms that differ in the robustness of a system they contain (white and black circles). Mutations are less likely to be deleterious in a robust system, and organisms that carry such robust systems thus survive preferentially.

generations, carriers of the more robust system survive preferentially. Thus, natural selection does not act on fitness differences among systems of different robustness. It acts only on the fitness differences caused by mutations in these systems. This simple insight has an important consequence: Selection for robustness in response to mutations is usually weak. Before I explain why, I need to give some necessary background information from the well-supported neutral theory of molecular evolution (222).

### Neutral Molecular Evolution

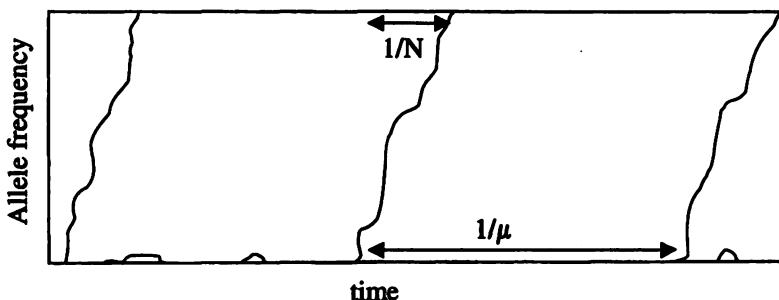
Imagine a population of  $N$  organisms containing some system, say some protein whose function is necessary for the survival of each individual. Assume that all individuals harbor identical genes for this protein, whose robustness is thus the same among all individuals. Random mutations that do not affect the protein's function—but may change its robustness—occur at a small rate  $\mu_n$ , say,  $10^{-6}$  mutations per gene, individual, and generation. What is the fate of such mutations? The neutral theory of molecular evolution shows that they have a probability of  $(1/N)$  to go to fixation, that is, to attain a frequency of one in the population (285). Because these mutations are neutral, only random genetic drift—the sampling of individuals from one generation to the next—accounts for their fixation. If such neutral mutations go to fixation, they take on average  $N$  generations to do so. (The factor  $N$  that occurs here and below holds for haploid organisms, and simply needs doubling to  $2N$  for diploid organisms.)

Because the population has  $N$  individuals, in every generation  $N\mu_n$  neutral mutations occur in the population on average. Each of these neutral mutations goes to fixation with probability  $(1/N)$ . Thus, the rate at which neutral mutations arise that will eventually go to fixation calculates as  $(N\mu_n)(1/N) = \mu_n$ . Put differently, one has to wait on average  $1/\mu_n$  generations until a mutation arises that will go to fixation. (This may be a very long time, of the order of a million generations if  $\mu_n = 10^{-6}$ .) That this waiting time depends only on the neutral mutation rate is perhaps the most celebrated result of the neutral theory of molecular evolution (285). It provides the foundation for large fields of modern biology such as molecular systematics.

With this background information in mind, consider first a small population or a population with a small mutation rate, such that the product of  $N$  and  $\mu_n$  is much less than one ( $N\mu_n \ll 1$ ). For instance, in a population of  $N = 100$  individuals where  $\mu_n = 10^{-6}$ , it takes on average one million generations for any mutation to occur that then goes to fixation—in a small number of  $N = 100$  generations (Figure 16.2a). For most of the time—almost one million generations—between the occurrence of two such

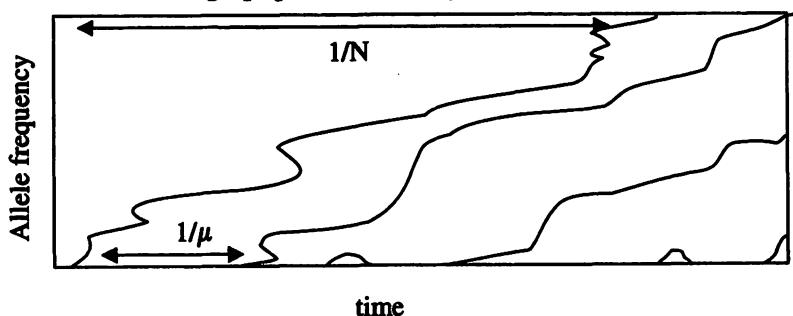
a)

Small population or low mutation rate



b)

Large population or high mutation rate



**Figure 16.2** Evolution of neutral variants of a system (which may differ in their robustness). (a) Small populations or organisms with small mutation rates ( $N\mu \ll 1$ ) are monomorphic most of the time. Neutral mutations that go to fixation arise only rarely and go to fixation quickly. (b) Large populations or organisms with high mutation rates ( $N\mu \gg 1$ ) are polymorphic most of the time. Neutral mutations that go to fixation arise frequently and take a long time to go to fixation. Selection for robustness, as shown in Figure 16.1, can increase population robustness only in a polymorphic population. After (285).

mutations, such a small population will be monomorphic. That is, all individuals will carry identical alleles of the gene. Specifically, the probability that two individuals picked at random from a population carry different alleles can be calculated as  $2N\mu_n/(2N\mu_n + 1)$ , which is very close to zero if  $N\mu_n \ll 1$  (222).

In a large population, or in an organism with a large mutation rate, such that  $N\mu_n \gg 1$ , the situation is fundamentally different. First, the

probability that two individuals chosen at random from the population carry different alleles approaches one with increasing  $N\mu_n$ . The reason is that now the waiting time  $1/\mu_n$  for mutations that will eventually go to fixation is short compared to the number of generations  $N$  that it takes the mutation to go to fixation (Figure 16.2b). During these  $N$  generations, there are at least two alleles in the population. In addition, other mutations can arise during this time that rise to appreciable frequency before becoming extinct again. Put differently, populations where  $N\mu_n \gg 1$  will be polymorphic most of the time.

### Selection for Robustness Is Usually Weak

To see what all this has to do with the evolution of robustness, consider that natural selection (of anything) needs genetic variation within a population. Thus, natural selection of robustness needs variation in robustness. Put differently, only in populations polymorphic for robustness can selection change robustness. A variety of studies arrive at this basic insight—the importance of variation for the evolution of robustness—from different angles (448, 562, 579, 582, 585, 591). The simple caricature of Figure 16.1 incorporates this insight, because its upper panel shows a population that is polymorphic (white and gray circles) for alleles that confer different robustness on a system. Such a polymorphism—any polymorphism—is frequent only in a population with  $N\mu_n \gg 1$ . It is exceedingly rare in a population with  $N\mu_n \ll 1$ . In addition, selection can only distinguish systems with different robustness if mutations affect them, mutations that have deleterious effects in the less robust system and that are neutral in the more robust system (Figure 16.1). Again, the likelihood that such mutations occur while a population is polymorphic for robustness increases with population size and mutation rate. It is large only for  $N\mu_n \gg 1$ .

Thus, selection for robustness as an adaptation to mutations is strong only if population sizes are large or if mutation rates are high. Put differently, the selective advantage of robustness is of the order of the mutation rate. Population sizes must be greater than the inverse of the mutation rate for natural selection to affect robustness. Clearly, the range of organisms for which these conditions hold will be limited. Good candidates are small organisms with enormous population sizes, or some viruses, such as RNA viruses with mutation rates hundred times higher than those of DNA-based organisms (123).

Everything I have said thus far, and much of what I will say below holds on or below the level of individual genes or protein. What about other levels of organization, such as that of genetic networks comprising

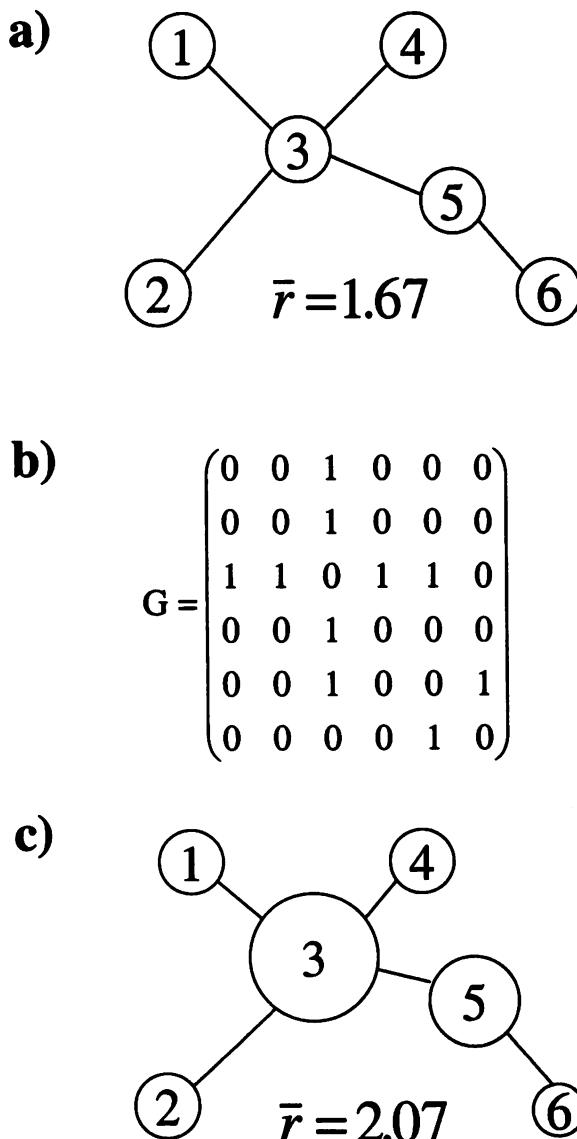
few to thousands of genes? Systems at different levels of biological organization differ critically in the rate at which mutations affect them. If—as I do here—one defines  $\mu_n$  as a mutation rate per gene, then an  $n$ -gene system suffers mutations at a rate proportional to  $n$ , e.g.,  $n\mu_n$ . Concomitantly, the probability that the population is polymorphic for variants of the system increases with the number of genes, and selection for robustness can become effective at smaller population sizes.  $N\mu_n$  no longer has to be greater than 1; it suffices that  $N\mu_n$  is greater than  $1/n$ . The more genes there are in a network, the more effective selection for robustness is, simply because there will be more variation in robustness (448, 591).

In sum, two general principles govern the evolution of robustness as an adaptation to mutations. First, natural selection acts indirectly, through the differential effects of mutations on systems with different robustness. Second, natural selection requires polymorphism in robustness, which is most likely to exist in large populations, organisms with high mutation rates, and systems consisting of many genes. These principles hold for all biological systems. I now present two more specific examples to illustrate how these principles can manifest themselves differently in different systems. For maximum contrast, these examples discuss the two different mechanistic causes of robustness, distributed robustness and redundancy of parts (chapter 15). Some of the following material is quite mathematical, but the main results are encapsulated in Figures 16.3 and 16.5.

### Evolution of Robustness in Discrete Neutral Spaces

A paradigmatic case for distributed robustness is the robustness of RNA structures to mutations (chapter 4), where a general framework to explain the evolution of robustness exists (55, 562, 608). Importantly, this framework applies not only to RNA secondary structure, but to any system that can be represented in a discrete neutral space, including proteins, the genetic code, and genetic networks that lend themselves to a discrete representation. In presenting this framework (55, 562, 608), I follow the simple yet elegant treatment of van Nimwegen and collaborators (562).

Consider a set of genotypes that all have the same phenotype. For visualization, it may be easiest to focus on a particular kind of genotype, such as RNA or protein molecules of length  $l$  that fold into the same structure, a structure required for some biological process. These genotypes form a neutral space, which is aptly called a neutral network (491) if all genotypes in the space are connected. In this case, one can represent this neutral space as a connected graph  $G$ , a mathematical object consisting of nodes (genotypes) and edges (Figure 16.3a). In this graph, two nodes



**Figure 16.3** The structure of a discrete neutral space completely determines to what extent natural selection can increase robustness. (a) The circles symbolize six hypothetical genotypes that are connected in a simple neutral network, displayed here as a graph. The average robustness of genotypes is the average number of one-mutant neighbors a genotype has. This average robustness calculates here as  $(1 + 1 + 4 + 1 + 2 + 1)/6 = 1.67$ . (b) The adjacency matrix, an alternative representation of the graph in (a). (c) A large population will reach a steady-state robustness

(genotypes)  $g$  and  $h$  are connected by an edge if they are neighbors, that is, if they can be reached from each other by a single change of a nucleotide, amino acid, or some other system part. For a macromolecule consisting of  $l$  amino acids or nucleotides, at each of whose positions  $a$  different building blocks are possible ( $a = 20$  for proteins), each genotype has  $l(a - 1)$  immediate neighbors, only some of which will be part of the neutral space.

The purpose of the following paragraphs is to show that the maximally attainable robustness in any such system depends *only* on the structure of the graph  $G$ . To begin with, imagine a very large (infinite) evolving population of identical genotypes. Their fitness  $w$ —no matter how defined—is identical because they have the same phenotype. For clarity of the argument, assume that these genotypes are optimal in some suitably defined sense. That is, their fitness cannot be improved by mutation. In every generation, individual members of the population suffer mutations—with a probability  $\mu$ —that change one nucleotide, amino acid, or some other system part. As a result of mutations, some genotypes are no longer part of the neutral network. That is, they adopt an inferior phenotype and get eliminated from the population. Other genotypes remain on the neutral network after mutation. These are genotypes that have undergone a neutral mutation. Such mutations occur at a rate  $\mu_n \leq \mu$ . The population will eventually reach a steady state where the mean fitness  $\bar{w}$  of the population no longer changes (95). In this state, an unchanging proportion  $P$  of the population is part of the neutral network. However, even in this steady state, selection and mutation continue to act. Specifically, natural selection will change, in each generation, the proportion of individuals that are part of the neutral network to  $wP/\bar{w}$  (95). Because of mutations, only a fraction  $\mu_n$  of these survivors will remain on the network. In the steady state, the population thus must fulfill the following equation:

$$\frac{\mu_n w P}{\bar{w}} = P \quad \text{or} \quad \frac{\mu_n w}{\bar{w}} = 1 \quad (16.1)$$

---

equivalent to the largest eigenvalue of the matrix in (b), as defined in equation 16.7. This eigenvalue calculates as  $\tilde{r} = 2.07$ , meaning that genotypes in the population have on average slightly more than two neighbors. This evolved robustness is 24% higher than the average robustness of 1.67 in the network. The population achieves this higher robustness by becoming concentrated at highly connected—robust—genotypes. The fraction of individuals in the population with a specific genotype is indicated by the size of the circles. Clearly, more individuals have the robust genotype 3 than the less robust genotype 6. Specifically, the proportion of individuals with genotypes 1–6 are approximately 0.14, 0.14, 0.28, 0.14, 0.18, 0.09, respectively. This proportion can be determined from the leading eigenvector of the matrix in (b) (562).

This treatment neglects the rate at which genotypes that are not part of the network mutate onto the network. To do so is appropriate if mutation rates are small, such that most of the population resides on the network, and if genotypes outside the network have fitness much lower than  $w$ .

To understand the evolution of robustness in this scenario, one needs to quantify a *population's* robustness. The most important quantifiers of population robustness derive from the robustness  $r_g$  of a genotype  $g$ . For any genotype  $g$ ,  $r_g$  is simply the number of its immediate neighbors that have the same phenotype, that is, the number of neighbors that are also in the network. If a proportion  $P_g$  of the population has genotype  $g$ , the average population robustness calculates as

$$\bar{r} = \sum_g r_g \left( \frac{P_g}{P} \right) \quad (16.2)$$

The quantity  $\bar{r}$  is closely related to the average rate of neutral mutations  $\mu_n$ . To see this, first note the following. If the probability that a genotype  $g$  is affected by a neutral mutation is given by  $\mu_{ng}$ , then the average rate of neutral mutations in the population calculates as

$$\mu_n = \sum_g \mu_{ng} \left( \frac{P_g}{P} \right) \quad (16.3)$$

Second, if mutations affect all  $l$  monomers (system parts) with equal probability, then the probability that a mutation in genotype  $g$  is neutral is  $r_g/l(a-1)$ , where  $l(a-1)$  is the total number of one-mutant neighbors of the genotype. Because mutations occur at a rate  $\mu$ , one can easily show that

$$\mu_{ng} = 1 - \mu \left( 1 - \frac{r_g}{l(a-1)} \right) \quad (16.4)$$

Equation 16.4 shows that a genotype's robustness  $r_g$  can be expressed as a function of its neutral mutation rate  $\mu_{ng}$  and vice versa. Together with (16.2) and (16.3), it also shows that a population's average robustness  $\bar{r}$  can be expressed in terms of the average neutral mutation rate  $\mu_n$  and vice versa. In this sense, average robustness and average neutral mutation rate are equivalent quantifiers of the same phenomenon.

I note in passing that the population's robustness can also be expressed in terms of its mean steady-state fitness (562). Expression 16.1 showed that in the steady state the neutral mutation rate  $\mu_n$  can be viewed as a function

of mean fitness. Using (16.1), one can substitute (i)  $\bar{w}/w$  for  $\mu_n$  in (16.3), and (ii) the right-hand side of (16.4) for  $\mu_{ng}$  in (16.3), and show that

$$\bar{r} = l(a-1) \left( 1 - \frac{w - \bar{w}}{\mu w} \right) \quad (16.5)$$

Thus, the population's mean robustness in steady state is a linear function of the population's mean fitness.

So far, I have shown how population robustness and neutral mutation rates are different ways of viewing the same phenomenon, and that population robustness becomes a simple function of population fitness in steady state. The structure of the network graph  $G$  has not even entered the consideration. This structure, however, becomes important in the next step. Analogous to the steady-state equation (16.1) for the fraction  $P$  of a population on the neutral network, one can establish a more complicated condition for the frequency  $P_g$  of each different genotype in steady state (562). This condition reads as

$$P_g = (1 - \mu) \frac{w}{\bar{w}} P_g + \sum_{h \in [g]} \left( \frac{w}{\bar{w}} \right) \left( \frac{\mu}{l(a-1)} \right) P_h \quad (16.6)$$

The leftmost term on the right-hand side corresponds to the fraction  $P_g$  of genotypes  $g$  that survive (with probability  $w/\bar{w}$ ) and that do not undergo mutation (probability  $1 - \mu$ ). The right-most term sums over all genotypes  $h$  on the neutral network that are one-mutant neighbors of  $g$ . The set of these genotype is indicated by the symbol  $[g]$ . More specifically, this term sums over all such genotypes that survive (with probability  $w/\bar{w}$ ), undergo mutation (with probability  $\mu$ ), and where this mutation turns them into genotype  $g$ , one of their  $l(a-1)$  one-mutant neighbors (with probability  $1/l(a-1)$ ). Genotypes outside the neutral network are again neglected.

One can simplify equation 16.6 by collecting all terms different from the summation over  $P_h$ ,  $\sum P_h$ , on the left-hand side of the equation. A few simple manipulations show that the left-hand side then becomes equivalent to  $\bar{r}P_g$ . The resulting equation (562) is then most compactly expressed using matrix notation

$$G\vec{P} = \bar{r}\vec{P} \quad (16.7)$$

Here,  $\vec{P}$  indicates a vector whose entries  $P_g$  corresponds to the proportion of the population in steady state that has genotype  $g$ .  $G = (g_{ij})$  is a binary

square matrix: A matrix element  $g_{ij}$  is equal to one only if the genotypes  $i$  and  $j$  are on the neutral network and if they differ by only one point mutation. It is zero otherwise. This matrix is also known as the adjacency matrix of the graph defining the neutral network (Figure 16.3b). It completely defines this graph.

In steady state, where the forces of mutation and natural selection balance, any population has to meet (16.7). The equation specifies which proportion  $P_g$  of the population resides at genotype  $g$ . Therefore, it also specifies the average robustness  $\bar{r}$  of the population in steady state. Specifically, the Frobenius–Perron theorem of matrix algebra (42) shows that if all genotypes are connected in the graph  $G$ , then the population's equilibrium robustness is equivalent to the largest eigenvalue of its adjacency matrix  $G$  (562). The genotype frequencies  $P_g$  are completely determined by the eigenvector corresponding to this eigenvalue.

Figure 16.3 illustrates an application to a simple, hypothetical neutral network consisting of only six genotypes. A genotype in the network has on average 1.67 neighbors (Figure 16.3b). The adjacency matrix of the network is shown in Figure 16.3b. Its largest eigenvalue calculates as  $\bar{r} = 2.07$ , meaning that a genotype in a steady-state population has on average 2.07 neighbors in the network. This mean population robustness is 24% higher than the average robustness, 1.67, of a genotype. Overall, this means that a large evolving population becomes concentrated at highly connected robust genotypes. The proportion of the population that has a particular genotype can be calculated from the adjacency matrix and is indicated by the size of the circles in Figure 16.3c. While the above derivation of (16.7) involves some approximations, its basic results agree with numerical work on the evolution of structural robustness in RNA molecules (562).

The generality of this result cannot be overstated. First, note that the equilibrium robustness attained in the population is independent of the mutation rate. It is also independent of the fitness of the genotypes in the neutral network. It is independent of the mean population fitness. And it is independent of the population size, as long as  $N\mu \gg 1$ . It depends only on the structure of the neutral space, which is completely defined by  $G$ . This again underscores the key importance of understanding neutral space structure to understand evolved mutational robustness. Although it is easiest to derive this result with a concrete genotype–phenotype relation in mind, such as that of RNA sequences and their spatial structure, the result transcends any such specific relation. It applies to any connected graph  $G$ , and thus to any genotype–phenotype relation that can be represented in this framework. It can also be generalized to cases where the neutral space does not consist of one but of several disjoint neutral networks (608). In addition, its results could be extended to cases where

other agents of genetic change, such as reciprocal recombination, are more important than point mutations. In this case, neighboring genotypes would correspond to genotypes that can be transformed into each other by the agent of change.

All this also shows that it is not possible to answer the question how much selection can increase robustness in general terms. If a network graph has a small number of very highly connected genotypes, then robustness can increase greatly. If the same graph has more uniform connectivity, it can increase very little.

What are the limitations of this approach? First, it applies only to systems that are conveniently represented in a discrete space. Genetic and metabolic networks, for instance, are not such systems. Consequently, we currently do not have a similarly simple framework for them. Second, the approach may break down where genotypes outside a neutral network have only slightly lower fitness, or where mutation of genotypes outside the network cannot be neglected.

The third and most important caveat regards the structure of the graph  $G$ . Although its properties are easily determined for hypothetical examples (Figure 16.3) and for small macromolecules, neutral spaces are often vast. This will often make it difficult to determine the complete structure of a graph  $G$ , and thus the maximal robustness a system can attain. However, advances in the analysis of large graphs may lead to improvements in estimating graph structure from incomplete information (6), and another possible remedy comes from an observation I made above: Mean robustness shows simple relationships to global population parameters, for which knowledge of  $G$  is not necessary. Equation 16.5 exemplifies one such relationship. The equation shows that a population's mean fitness is linearly related to the mutation rate, with a slope that is a function of mean robustness  $\bar{r}$ . In vitro evolution approaches, which are very successful at generating biological molecules with new activities (137, 263, 318, 488, 558), could be used to estimate how a molecular population's mean fitness depends on the mutation rate, and thus provide an estimate of  $\bar{r}$  without information about  $G$ . Similarly simple relationships have been observed for other parameters, such as the fraction  $P$  of a population that is part of a neutral network and robustness  $\bar{r}$  (608).

### Populations with $N\mu \ll 1$

All of the above results hold for very large populations ( $N\mu \gg 1$ ). How do small populations ( $N\mu \ll 1$ ) differ? Recall that such populations are monomorphic most of the time, i.e., all its members have the same genotype  $g$ . Every  $1/\mu_{ng}$  generations, a neutral mutation to a genotype  $h$  arises

in the population, a mutation that goes to fixation rapidly, in about  $N$  generations. At this point, the entire population becomes monomorphic again and stays monomorphic until the next neutral mutation arises. The population has effectively moved from genotype  $g$  to genotype  $h$ .

Over a long time, a small population thus effectively performs a random walk on the network  $G$ . The mathematical theory of random walks shows that such a population will spend equal amounts of time at each node (562). Thus, the population's robustness becomes the average robustness over all genotypes  $g$  in the network. Put differently, the population cannot become concentrated at genotypes of high connectivity and robustness. In terms of the simple example of Figure 16.3, the population's robustness would be equivalent to the robustness of genotypes on the network (1.67, Figure 16.3a), and not the higher robustness of 2.07 in Figure 16.3c. In this sense, small population sizes prevent the evolutionary increase of robustness, as I discussed above in more general terms. For populations of intermediate sizes or intermediate mutation rates, evolved robustness becomes intermediate between the lower limit of  $N\mu \ll 1$  and the upper limit of  $N\mu \gg 1$ .

The above arguments show that some time-honored principles of population genetics, such as the relationship between polymorphism and population size, are of great value in understanding the evolution of robustness. In contrast, they render other such principles untenable (562, 610). One of them is the famed Haldane-Muller principle (222), which states that the mean fitness of a population in mutation-selection balance depends only on the mutation rate. Equation 16.5 shows that this principle is violated here. Mean fitness depends on the mutation rate, but also on the mean robustness  $\bar{r}$  of a population, which is a function of neutral space structure. This serves as a cautionary note that population genetic intuition—which usually does not take the architecture of biological systems into account—may fail where this architecture becomes key.

## Partial Gene Redundancy

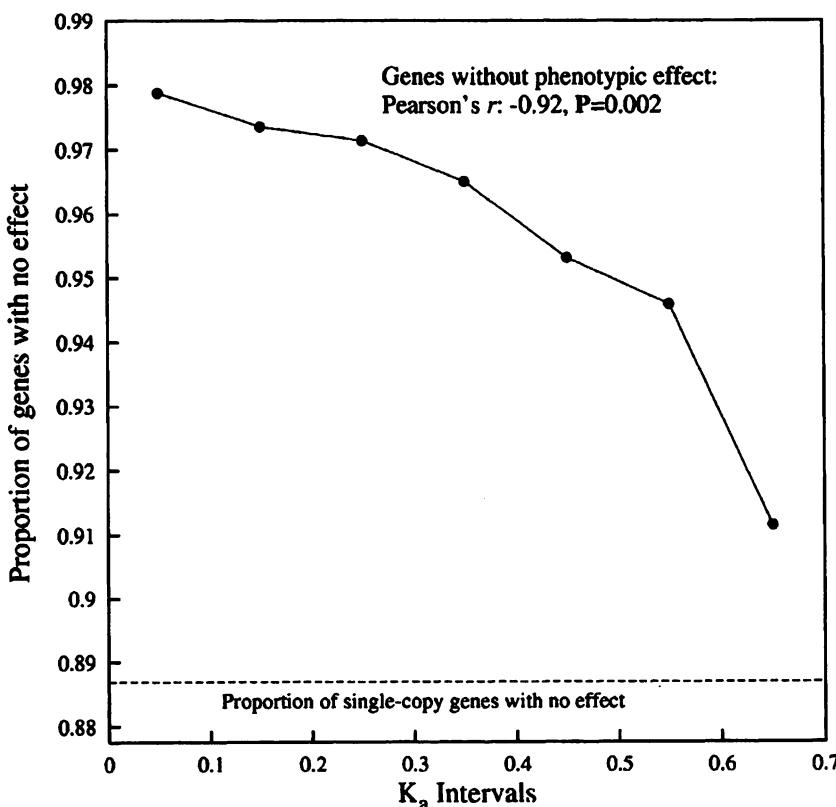
Eukaryotic genomes typically contain thousands of genes whose elimination has no detectable effect on the organism. As I argued earlier, distributed robustness is an important cause of this phenomenon, but gene redundancy also plays a role in it (chapter 15). One might suppose that such redundancy is always a transient phenomenon in evolution: Two duplicate genes may be fully redundant only shortly after their duplication. As time passes, one of the duplicates becomes silenced or they diverge in their function (12, 63, 165, 348, 394, 408–410, 580, 581, 583, 597, 602, 622). And, indeed, many duplicate genes show signs of rapid functional

divergence (chapter 15). However, if such divergence was the fate of all duplicate genes, then only recently duplicated—and thus highly similar genes—would have no effect when eliminated from a genome. The facts show otherwise. I discussed in chapter 15 gene families such as the *TPK* and *CLN* genes in yeast. The members of these gene families have similar function but derive from ancient duplications that occurred more than 100 million years ago.

Ancient gene duplicates with similar function are not rare exceptions to a rule. Their signature is visible even in genome-wide analyses of gene inactivation effects. Figure 16.4 shows the results of a pertinent analysis of more than 13,000 genes of the nematode worm *C. elegans*, and of the effect of their temporary inactivation through interference with their mRNA expression (RNA interference) (268). In this analysis, Gavin Conant and I (86) grouped gene duplicates into categories according to their amino acid divergence, as measured via the fraction  $K_a$  of amino acid replacement substitutions at amino acid replacement sites (horizontal axis) (336). We then plotted, for each divergence category, the proportion of genes whose inactivation has no detectable phenotypic effect, and compared it to the proportion of single copy genes with no detectable effect (dashed line). The data show that even for the most highly diverged gene duplicates ( $K_a > 0.6$ ), temporary gene inactivation is more likely to result in no effect than for single-copy genes ( $K_a > 0.6$ ). Most of these highly diverged duplicates have no detectable sequence similarity at the silent nucleotide sites that are an indicator of duplication age (86, 336). This means that many ancient gene duplicates, duplicates so ancient that their divergence time can no longer be estimated, still have overlapping functions.

How are gene duplicates with similar functions maintained long after duplication? There are at least two possibilities. First, some duplicates may be preserved because it is advantageous for the organism to produce more of the gene's product. Candidates are very highly expressed genes, such as those encoding ribosomal DNAs. The elimination of such duplicates, however, would not go unpunished, because lowering gene expression by eliminating one gene copy would have deleterious effects. Thus, duplicates maintained for this reason have no role in mutational robustness.

A second possibility is that gene duplicates are maintained for adaptive reasons, that they protect an organism against otherwise deleterious mutations. Here, it is important to note that this is not possible for genes with only one function. The reason is that mutations would eventually eliminate this function for one of the duplicate genes, and thus silence the gene (305, 404). However, most genes are multifunctional (chapter 14). Multifunctionality is key for maintaining gene duplicates in a genome (165, 305, 404). To see this, consider a mutation that eliminates merely one of a gene's many functions. This mutation renders a duplicate of the



**Figure 16.4** Even highly diverged duplicate genes are more likely to show no phenotypic effect in RNA interference assays than single-copy genes (86). On the horizontal axis, duplicates are binned according to their amino acid divergence  $K_a$  (336). The vertical axis shows the proportion of genes belonging to pairs in each divergence category, whose temporary silencing through RNA interference has no phenotypic effect. The age of duplicates cannot be reliably estimated from amino acid divergence. However, for a third of the 4639 duplicate genes analyzed for this figure, synonymous sites on DNA have completely diverged, demonstrating that these duplicates are ancient (86).

gene essential, because the duplicate then becomes the only gene performing this function. By the same token, gene duplicates in which different functions have gotten lost must both be maintained in a population. Multifunctionality also implies that two genes may diverge only in some of their functions.

Unfortunately, it is impossible to quantify exactly to which extent two duplicate genes overlap in their functions, simply because it is impossible

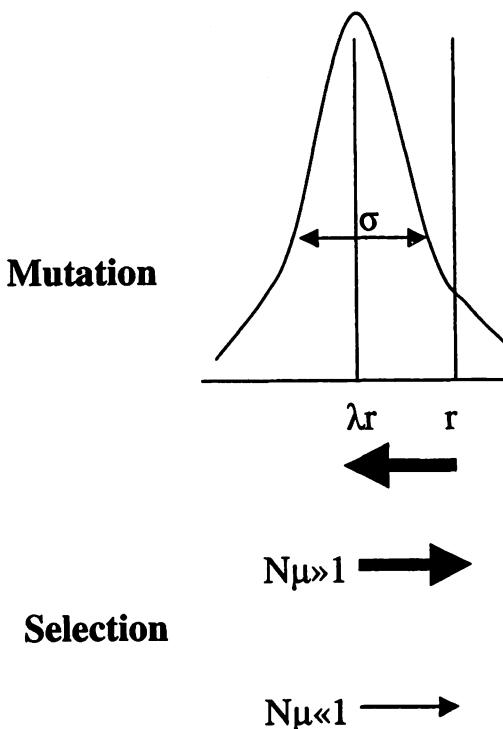
to characterize any gene's functions exhaustively (chapter 14). However, one can estimate the overlap through various indicators of function, such as similarity in gene expression pattern, similarity in common interactions with proteins or DNA, or the number of transcriptional regulators regulating the activity of both gene products (143, 583, 586, 587). There is also ample experimental evidence, some of which I discussed in chapter 15, that duplicate genes can differ in some functions, yet share others.

In light of these observations, the task is to explain how multifunctional genes with overlapping or partially redundant functions can be maintained through the adaptation to mutations they provide. I now review a simple model that does just that (582, 585). This model is best viewed as conceptual in nature, because key model parameters have not yet been measured. At this point, the model serves to illustrate the evolutionary mechanism maintaining overlapping gene functions. Its assumptions are simple and biologically sound and its results hold under very general conditions, which suggests that it captures some of the reality of how overlapping gene functions evolve.

### A Model for the Maintenance of Partial Redundancy

If two genes are multifunctional, then the functional overlap among them can be approximated by a variable  $r$  that assumes values between zero and one. Two genes with  $r = 1$  are completely redundant—maximally robust to loss-of-function mutations—and two genes with  $r = 0$  have completely different functions. Partial redundancy or functional overlap ( $0 < r < 1$ ) corresponds to an intermediate state where some mutations have no deleterious effect. In the simplest possible scenario, the probability that a mutation has no deleterious effect is linearly related to redundancy  $r$ . That is, if mutations affect one of the genes at rate  $\mu$ , then the probability that a mutation is neutral is given by  $\mu r$ . Conversely, the probability that the mutation is deleterious is  $\mu(1 - r)$ . (If  $r$  is not linearly related to robustness, then one can always find a transformation of  $r$  where linearity holds.) While it is possible to study the effect of beneficial mutations on redundant genes (593), the evolution of robustness is most easily understood by considering only neutral and deleterious mutations.

Even phenotypically neutral mutations can eliminate one or more functions of a gene, as long as these functions are also exerted by the other gene. Because a mutation is more likely to eliminate a gene function than create a new one, the net effect of mutations is a reduction of the functions exerted by both genes, that is, a reduction in  $r$ . Specifically, I will assume that a mutation reduces  $r$  on average to some value  $\lambda r$ , where  $0 < \lambda < 1$ . Not every mutation may change  $r$  to the same extent, however. This



**Figure 16.5** The evolution of overlap (partial redundancy)  $r$  in gene functions. On average, mutations reduce overlap by some factor  $\lambda$ , because most mutations eliminate one or more functions of a multifunctional gene. In large populations ( $N\mu \gg 1$ ), selection can counteract this mutational decay of functional overlap, because mutations in gene pairs with high  $r$  are less likely to have deleterious effects. Put differently, gene pairs with high  $r$  survive preferentially. A balance is achieved when the population's mean redundancy has reached a value of  $\bar{r} = \sqrt{\sigma^2 \lambda / [2\lambda^3 - 3\lambda^2 + 1]}$ . In small populations ( $N\mu \ll 1$ ), polymorphisms in functional overlap are so rare that selection cannot sustain overlap indefinitely. That is, selection cannot counteract the diversifying effect of mutations and redundancy thus slowly decays to zero (582, 585).

possibility is best represented by a standard deviation  $\sigma$  of the mutational change in overlap (Figure 16.5). No further assumptions about the distribution of the reduction in  $r$  are necessary for the results I discuss below.

The mutational effect of reducing redundancy is counteracted by natural selection. That is, mutations are more likely to have a deleterious effect for gene pairs with lower  $r$ , which will cause the preferential elimination of their carrier organisms. As for the earlier example of discrete neutral

spaces, this effect of selection is indirect, acting not through superior fitness of gene pairs with higher  $r$ , but through the reduced likelihood of deleterious mutations in them (Figure 16.1).

The quantifier  $r$  is analogous to the robustness  $r$  of a genotype in a discrete neutral space, and one could represent families of duplicate genes in such a space (chapter 15). However, the mathematical model of the previous section does not immediately apply, because mutations no longer transform different genotypes into each other at equal rates: Mutations are more likely to lead to losses of functions and thus to a reduction of  $r$ .

The mutational reduction of redundancy  $r$  and its increase through selection are opposing evolutionary forces. The net result is a balance in which some redundancy is maintained ( $r > 0$ ). Specifically, one can show (582) that under the above assumptions and when  $N\mu \gg 1$ ,  $r$ , averaged over a population, reaches a steady-state mean value that depends only on  $\lambda$  and  $\sigma$ . This value can be calculated analytically as  $\bar{r} = \sqrt{\sigma^2 \lambda / [2\lambda^3 - 3\lambda^2 + 1]}$ . For instance, if mutations reduce functional overlap on average by 20% ( $\lambda = 0.8$ ) with a variance of  $\sigma^2 = 0.1$ , then natural selection can maintain an average functional overlap of  $\bar{r} = 0.88$ . This means that 88% of mutations that would be deleterious if the gene had only one copy are neutral.

This formula is of limited practical use, because nobody has estimated  $\lambda$  and  $\sigma$  for any two genes (582). It merely goes to show that natural selection can maintain gene redundancy in a population. It does so under very general conditions that are independent of the mutation rate  $\mu$ ; insensitive to the extent to which a deleterious mutation decreases fitness; and independent of whether two genes are linked or not. The result holds also for families of  $n > 2$  duplicate genes, if  $\bar{r}$  is defined as the average functional overlap between any two genes in the family. It is also independent of the population size, as long as  $N\mu \gg 1$  (582, 585).

### What Happens in Populations with $N\mu \ll 1$ ?

Again, bear in mind that a population will be monomorphic most of the time if  $N\mu \ll 1$ . Thus, every individual in the population has the same  $r$ , and thus the average redundancy  $\bar{r}$  in the population, is identical to  $r$ . The rare neutral mutation that goes to fixation reduces, on average, an individual's redundancy by a factor  $\lambda$ . After this mutation has gone to fixation, the population's mean redundancy  $\bar{r}$ , will thus have also been reduced by a factor lambda, i.e.,  $\bar{r} = \lambda r$ . When the next neutral mutation arises that goes to fixation, the process repeats. Thus, the average population redundancy suffers a ratchet-like decrease by a factor  $\lambda$  every time a neutral mutation goes to fixation. The end result of this process is that gene

functions diverge completely. The following differential equation (585) describes the rate of this divergence over time  $t$ :

$$\frac{d\bar{r}}{dt} = (\log_e \lambda) \frac{2\mu\bar{r}^2}{(1 - 2\mu) + 2\mu\bar{r}} \quad (16.8)$$

Strictly speaking,  $\bar{r}$  here corresponds to the average redundancy not in one population, but in a large ensemble of populations, because the random effects of genetic drift prevent prediction of evolution's outcome in any one small population (95). Equation 16.8 has an important feature that the above verbal explanation did not capture. In the absence of selection, mutations would lead to an exponential decline of functional overlap, according to the differential equation  $d\bar{r}/dt = (\log_e \lambda)2\mu\bar{r}$ . This equation is similar to equations describing many exponential decay processes such as radioactive decay. However, in (16.8) the decay in  $r$  is not exponential but much slower—polynomial—because of the additional term  $\bar{r}/(1 - 2\mu + 2\mu\bar{r})$  on the right-hand side of (16.8). Here is why. If an individual with overlap  $r$  undergoes a mutation, only a fraction  $r$  of the mutants will survive. The remaining fraction,  $(1 - r)$ , will die. Put differently, for low values of  $r$ , neutral mutations become increasingly rare. Specifically, their rate is given by the term  $\bar{r}/(1 - 2\mu + 2\mu\bar{r})$  in (16.8). But neutral mutations are the only mutations that can have a lasting influence on redundancy in a population, because deleterious mutations get eliminated. Thus, a reduced rate of neutral mutations means a reduced rate at which gene functions diverge. Equation 16.8 reflects this reduced rate as a dependency of  $d\bar{r}/dt$  on  $\bar{r}^2$  instead of  $\bar{r}$ . In sum, in small populations, gene functions inevitably diverge, but this process becomes very slow when two genes have diverged considerably.

This deceleration of functional divergence among genes is even more drastic if many mutations have pleiotropic effects, that is, if they affect more than one function of a gene. To see why, consider two identical duplicate genes, where each of the genes carries out the same  $k$  functions. Over time, mutations eliminate some of these functions, such that only a fraction  $r = k_r/k$  of the functions are performed by both genes. If a mutation has pleiotropic effects—if it eliminates  $l > 1$  functions—then the probability that the mutation is neutral is no longer equal to  $r$  but equal to  $r^l$ . Put differently, the more extensive pleiotropic effects are, the less protection against deleterious mutations partial redundancy buys. In this case, the decay of redundancy in small populations depends not on  $\bar{r}^2$  but on  $\bar{r}^{l+1}$ . Specifically, 16.8 becomes

$$\frac{d\bar{r}}{dt} = \log_e \lambda \frac{2\mu\bar{r}^{l+1}}{(1 - 2\mu) + 2\mu\bar{r}^l} \quad (16.9)$$

How much does the decay of redundancy decelerate? To get at this question, define as  $T_m$  the time it takes to reduce mean functional overlap  $\bar{r}$  from one to  $(1/2)^m$ . Then, the difference  $T_m - T_{m-1}$  is the time it takes for each successive halving of  $\bar{r}$ . Equation 16.9 can be used to show that  $T_m - T_{m-1}$  is proportional to

$$\frac{2^{lm}(2^l - 1)}{l} \quad (16.10)$$

Expression 16.10 shows that the time necessary for successive halving of  $\bar{r}$  increases exponentially with pleiotropy  $l$ . To reduce redundancy in a population from  $\bar{r} = 1$  to  $\bar{r} = 1/4$  with a realistic genic mutation rate of  $2.5 \times 10^{-6}$  already takes more than  $10^7$  generations. If mutations are only moderately pleiotropic, such as if  $l = 3$ , this time increases by more than tenfold to approximately  $2 \times 10^8$  generations (585).

In sum, natural selection for robustness in small populations cannot sustain overlapping gene functions indefinitely. The divergence of gene functions through mutations is the dominant force in such populations. This illustrates the general principle outlined above that natural selection can sustain elevated robustness only in populations with  $N\mu \gg 1$ . However, natural selection can lead to a drastic, exponential slowing of functional divergence even in small populations, especially for genes that function in many different biological processes. This decelerated divergence of gene functions could explain why even highly diverged genes such as the worm genes of Figure 16.4 are more likely to have little phenotypic effect than single-copy genes.

### The Origins of Gene Duplications

All of the above regards the maintenance of gene redundancy. However, every gene duplication first arises in only one organism of a population. What makes it go to fixation in the first place, and is selection for mutational robustness essential for such fixation? For duplications of single genes, the answer is probably no. Single gene duplications occur as by-products of DNA replication, repair, recombination, and transposition events, processes that are an integral part of cellular life. They thus provide a population with a continuous trickle of gene duplicates. If this condition is met—if gene duplications occur continually, albeit at a small rate—then genetic drift is a sufficient cause to elevate many gene duplicates to fixation (81). Many gene duplicates, of course, may go to fixation because the increased gene number provides a direct advantage, such

as a greater amount of gene product. However, increased mutational robustness caused by gene duplications pales in comparison to drift and selection as a cause for the fixation of gene duplicates, especially because the advantage of robustness is weak in many populations.

A second major cause of gene duplication is polyploidization—the duplication of genomes—of which the transition from haploidy to diploidy is a special case. At face value, it would seem that polyploidy would be advantageous merely because it generates robustness through redundant copies of all genes. Unfortunately, the matter is not all that simple. For example, a diploid genome can contain many recessive alleles that would be lethal—and thus weeded out—in the haploid stage. From this perspective, an organism that spends part of its life cycle in a haploid stage, such as many plants, may have an advantage over a true diploid. Numerous studies show that whether a genotype with increased ploidy can become fixed because it provides an increase in robustness to mutations depends on a variety of factors, including the organism's life history, recombination rates, and the extent of dominance between alleles (299, 423, 424, 438). Other advantages of increased ploidy may thus be more important to establishing it in a population (350, 418, 421–423). They include favorable phenotypic effects of polyploidization, faster rates of adaptation in polyploids, and a more diverse set of gametes under circumstances where selection favors such variability.

## A Universal Theory?

The above two models regard two maximally different mechanistic causes of robustness: distributed robustness and redundancy of parts. It may thus not be surprising that there are many differences in how selection can maintain and increase robustness through these mechanisms. For instance, in large populations, the robustness of a population of biological macromolecules attains a mutation-selection equilibrium determined only by the connectivity of a neutral space. In contrast, for overlapping gene functions, this equilibrium depends only on the extent to which mutational pressure causes functional divergence. In small populations, the average robustness of a population of biological macromolecules is generally not zero, but given by the average robustness of all genotypes in a neutral network. In contrast, robustness caused by gene redundancy will eventually disappear completely in such populations, albeit sometimes at a glacial pace.

These differences suggest that there may be no fundamental theory of how robustness evolves, if such a theory is required to take into account the different architectures of biological systems. The reason is that the

mechanistic cause of robustness strongly influences how robustness can evolve. However, the absence of such a theory does not mean anarchy. Simple yet general principles can still apply to all biological systems. Two of these principles I have highlighted here: Selection for robustness is indirect. And it is strong only where much variation in robustness occurs, such as in large populations, in organisms with high mutation rates, or in genetic networks with many genes.

## Robustness as an Evolved Adaptation to Environmental Change and Noise

The previous chapter discussed the role mutations play in the evolution of robustness. This chapter focuses on an alternative explanation for the evolution of mutational robustness, an explanation that is really very simple. Mutational robustness can be a by-product of an organism's need to survive and reproduce in the face of *nongenetic* changes. These include changes in the organism's environment and continual changes inside the organism itself, for which the term noise is often used (22, 454).

At first sight, it seems self-evident that evolved robustness to mutations should be an adaptation to mutations. However, we saw that this notion faces a serious problem. Mutational robustness can evolve only under conditions where enough genetic variation in robustness exists. Few populations and biological systems meet these conditions. Only if population sizes are large, or if mutation rates are high, or if a system involves many genes will robustness vary sufficiently for it to increase in evolution. These limitations make the alternative explanation that I discuss here very attractive, because it applies under a much greater variety of conditions.

The central tenet of this chapter is that robustness to mutations can result from natural selection for robustness to nongenetic change. To prove this assertion requires positive answers to three questions. First—and this will be easy—is nongenetic change an abundant source of variation in the performance of biological systems? Second, and most important, are systems robust to nongenetic change also robust to mutations? Third, can natural selection readily modify robustness to nongenetic change?

### Nongenetic Change Affects All Aspects of Biological Systems

The first main source of nongenetic change affecting every organism is environmental change. Such change arises from chemical and physical factors, as well as from other organisms. I need to say little about its ubiquity. Temperature, water availability, soil conditions, photoperiod, nutrient availability, predator abundance, and many other aspects of an

organism's environment vary in space and time. Such variation affects every aspect of life.

A second kind of nongenetic change—noise—is equally ubiquitous but less appreciated, so I will say more about it. Even if it were possible to place organisms in a perfectly constant environment, they would still be subject to noise, because noise originates inside organisms themselves. The root cause of all internal noise is heat or thermal motion. From a thermodynamic perspective, heat is simply the random motion of particles, a motion that occurs at all temperatures above 0 K. It is essential to all life and is intimately tied to an important aspect of the environment: ambient temperature. (This simple observation shows that the two categories of nongenetic change—external change and internal noise—are not completely separable.)

The importance of thermal noise is implicit in many of the example I discussed earlier. Just take the folding of macromolecules like RNA and proteins, and their different conformations. Thermal noise causes the constant “wiggling” of these molecules between different conformations. As temperature increases beyond a molecule’s physiological optimum, the molecule is less and less likely to adopt its native conformation, a conformation that is usually necessary for proper functioning. Thermal noise can thus disrupt the function of biological molecules. Similarly, thermal noise is the root cause of mutations during DNA replication, and of translation errors during protein synthesis by ribosomes.

The effects of thermal noise manifest themselves on all levels of biological organization. Especially illustrative examples come from studies of gene expression (23, 49, 87, 138, 154, 296–298, 365, 366, 401, 425, 454, 564). Gene expression is not a deterministic but a stochastic process, a process strongly influenced by random events. Some of the earliest evidence for noisy gene expression came from the response of cell populations to increasing amounts of regulatory molecules. For example, increasing the concentration of the transcription factor NF-AT (nuclear factor of activated T cells) in T-cell populations increases gene expression in a stochastic manner. That is, in a population of T cells carrying a gene regulated by NF-AT, individual cells exposed to NF-AT do not express the gene at a higher level at any one time. Instead, some cells express the gene, whereas others do not, and only the proportion of cells that express the gene at any time increases with increasing amounts of NF-AT (154). Similar observations exist for other genes, such as the arginase gene in hepatocytes, and its expression response to glucocorticoid hormones (564), or for gene expression driven by the mouse mammary tumor virus regulatory region (298).

Noise in gene expression arises from both transcription and translation. The binding and release of transcriptional regulators and of RNA

polymerase on DNA are intrinsically random events (87, 296, 365). Similarly, the number of proteins produced from a messenger RNA before its degradation can vary greatly among identical messenger RNA molecules (365). At first sight, one might think that randomness in gene expression might be strongest for extremely lowly expressed genes, genes whose products exist at only a few copies per cell. However, this need not be the case. Several studies of prokaryotic and eukaryotic gene expression show that even highly expressed proteins show substantial amounts of noise (49, 138, 425). That is, among cells in a genetically uniform population, the standard deviation in the amount of a gene product can equal several times the mean product amount. Even one and the same cell containing two identical genes with identical regulatory regions can express these genes at greatly varying levels (138). The effects of such gene expression variation can cascade through networks of interacting genes (49, 138). Such gene interactions can greatly influence whether expression noise becomes amplified or damped as it reverberates through a gene network (49, 454, 544, 545).

Noise in gene expression is more than a biological curiosity: Not only is it ubiquitous, it can also have significant phenotypic consequences. One example is the “decision” of bacteriophage lambda to lyse its host cell—a response to host stress—or to remain dormant inside the host. This decision is driven by a gene expression network and involves randomness: Even in populations of genetically identical (stressed) cells and phages, only some cells undergo lysis, whereas others retain the dormant phage (23). Another example comes from a variety of human pathogens, such as *Neisseria gonorrhoeae*, responsible for gonorrhea, or *Vibrio vulnificus*, a bacterium causing gastrointestinal infections. Such bacteria can generate variation in their surface composition through a mechanism that exploits noise inside cells (454). This variation can help the bacteria evade the host’s immune response.

A third example, from the color patterns of butterfly wings, shows that phenotypic effects of noise are not restricted to single-celled organisms (401). Butterfly wing coloration serves important biological functions, such as predator avoidance. Coloration is genetically controlled: Different alleles at the same gene locus can cause differences in wing color in different parts of the wing. Importantly, boundaries between two areas of solid colors are usually not sharp but fuzzy. This fuzziness results from noise in pigment production. Specifically, individual pigment-expressing cells express either one or the other color, but not both. Similarly, butterflies heterozygous in a coloration gene often have a wing color intermediate between the colors of the homozygotes. The intermediate color, however, does not result from one pigmented cell expressing both color pigments. Instead, some cells express only one pigment, and others only

the other pigment. Because individual cells are very small, the color appears intermediate to our eyes. These phenomena reflect stochasticity in gene expression (401).

In sum, intrinsic noise influences biological systems on all levels of organization. This observation, together with an incessantly changing environment, indicates that all biological systems are subject to constant nongenetic change, which answers the first of the three opening questions.

### Nongenetic Change and Mutational Robustness: Above the Gene Level

I now turn to the second of the three opening questions: Does robustness to nongenetic change entail mutational robustness? The answer is, with few exceptions, yes (375). The two kinds of robustness are associated on multiple levels of biological organization. In the following paragraphs, I revisit relevant material from previous chapters, descending the hierarchy of biological organization.

Recall first the role of heat-shock proteins like Hsp90 in development (chapter 11). Malformations in multiple organismal characters—eyes and legs in fruit flies, roots and shoots in flowering plants—arise when the function of Hsp90 is impaired, either through mutations in its gene or through chemical agents that interfere with Hsp90. These malformations arise from genetic variation that is invisible and buffered when the protein is intact.

All of life, from bacteria to humans, depends on molecular chaperones, of which Hsp90 is one example. A main function of such chaperones is to assist in protein folding. That is, they prevent the aggregation of misfolded proteins, and they restore misfolded proteins to their native conformation. The source of protein misfolding is the thermal noise I discussed above, which increases with increasing temperatures. An earlier name for chaperones, heat-shock proteins, derives from their ability to protect organisms against heat stress: Cells regulate the activity or expression of these proteins in response to high temperatures. In addition, molecular chaperones also function to protect the organism against a variety of stressors, such as energy depletion, through mechanisms that are not fully understood.

In addition to these primary functions of chaperones, the example of Hsp90 shows that chaperones also happen to buffer proteins against mutations. It is easy to understand in principle how this works. Just consider a mutation that causes a folding defect in a protein. For instance, the unmutated protein may rapidly fold into its active conformation and spend 99% of its time in this conformation. In contrast, the mutated proteins may spend only 70% of its time in the native conformation. Chaperones may

coax the 30% of misfolded protein molecules back into their native conformation, and thus prevent the folding defect to ever become manifest phenotypically. In this way, proteins whose primary role is to provide protection against thermal noise serve the dual role of protecting against mutations.

A disadvantage of studying complex traits such as eyes and roots, traits whose formation is affected by Hsp90, is that our understanding of the relevant gene interactions is sketchy at best. However, the handful of developmental processes where such gene interactions are well understood reveal the same principle. For instance, the model of the segment polarity gene network in chapter 10 suggested that this network—necessary for subdividing a fly embryo into multiple segments—is robust to a variety of genetic changes. One of these changes is altered early expression of the genes *engrailed* and *wingless*, genes that activate the segment polarity network. Recall from chapter 10 that *engrailed* and *wingless* can be expressed in shallow gradients covering many cells, or in patterns without periodicity, and the segment polarity network may still produce the gene expression pattern necessary for proper development. Such changes in the spatial and temporal pattern of *engrailed* and *wingless* expression need not be caused by mutations. They may also be caused by gene expression noise or by variation in embryo sizes (243, 517), variation that can be caused by environmental change.

Thus, the segment polarity gene network is robust to variation in gene expression, variation that could be caused both by mutation and by nongenetic factors. This association is not a peculiarity of the segment polarity network. It is also manifest in a wider class of regulatory gene networks, networks that share an important property: cooperativity or synergism in gene regulation (chapter 10). Networks with many cooperative interactions exhibit and can evolve mutational robustness (579), but such networks robust to mutations are also robust to nongenetic change that temporarily alters the expression of individual genes (40). The same phenomenon holds for individual network genes whose expression or activity is regulated cooperatively. Recall that in cooperative gene regulation, gene activity changes drastically, sometimes switch-like, within a narrow range of a regulator's concentration. However, gene activity is insensitive to regulator concentrations outside this range. Whether changes in regulator concentrations occur through mutations that change the regulator's expression or its activity or whether they have nongenetic causes is irrelevant. Where cooperativity provides robustness against changes in regulator concentrations, it does so regardless of the cause of change. Thus, again, robustness to nongenetic change is associated with robustness to mutations.

Metabolic networks and pathways, completely different kinds of multigene systems, are no exception to this principle (chapters 8 and 9). The flux-balance analysis of complex metabolic networks shows that the

flux through individual chemical reactions can be changed drastically without affecting network performance. Similarly, the activity of individual enzymes in linear metabolic pathways can vary by more than 50% without affecting pathway flux. Again, only the changed enzyme activity is relevant, but not the cause of this change. For instance, a changed enzyme activity can be caused by a change in ambient temperature, but also by mutations that affect enzyme concentration.

Yet another example is that of gene redundancy. Duplicate genes with similar functions can provide robustness against mutations in one of the duplicates. However, redundancy can also provide robustness to random variation in gene expression, especially for the many genes that are expressed at a low level (218). Because gene expression is a stochastic process, the amount of product made from a lowly expressed gene may sometimes—by chance alone—fall below a minimum level necessary for a biological process. This may have detrimental consequences. Increasing gene copy number through gene duplication may prevent this problem, because it decreases the likelihood that gene expression falls below this threshold (87, 366, 401).

### Nongenetic Change and Mutational Robustness: On or Below the Gene Level

The next-lower level of organization regards individual macromolecules. Here, I focused earlier on the robustness of RNA and protein folding to mutations (chapters 4 and 5). At ambient temperature, many RNA and protein molecules have a unique most stable (minimum-free energy) structure. Through the influence of thermal noise these molecules adopt a spectrum of alternative, less stable structures. Mutationally robust RNA sequences—sequences whose most stable secondary structure is insensitive to many mutations—are also thermodynamically stable (chapter 4). That is, they are robust to thermal noise, which means three things. At any one temperature, robust structures adopt a smaller number of alternative structures, structures different from the most stable structure. Second, these alternative structures are more similar to the most stable structure. Third, these sequences spend more time in the most stable structure than less robust sequences. Similar observations hold for lattice proteins, simple models of protein folding (chapter 5). That is, mutationally robust proteins are also thermodynamically stable. Conversely, evolved thermodynamical stability implies evolved mutational robustness. Again, robustness to mutations is associated with robustness to nongenetic change.

Another case study below the gene level is the genetic code. The genetic code of extant organisms is more robust to mutations than the vast

majority of alternative codes, even when taking into account that its evolution was constrained by the evolution of amino acid biosynthesis pathways (chapter 3). Robustness of the code means that the average nucleotide substitution will change an amino acid to a chemically similar amino acid, thus minimally disrupting protein functions. Where does nongenetic change come in here? Nongenetic change is reflected in translation errors—caused by noise—that ribosomes occasionally make. Robustness to such translation errors means that a misread nucleotide will cause a chemically minimally disruptive amino acid change. Robustness to translation errors implies mutational robustness, and vice versa.

The lowest level of biological organization I discussed is that of the genetic material itself, and of its alphabet. In chapter 2, I surveyed evidence that the alphabet of today's genetic material is superior to many alternative alphabets: It better maximizes the binding energy of complementary bases while minimizing that of noncomplementary bases. This feature provides robustness to mutations that occur in DNA replication. Does this mutational robustness also provide robustness to noise? Yes, and most dramatically. Noise here comes from the DNA replication machinery, which must pick—from a pool of nucleotides—those complementary to the strand being replicated. A genetic alphabet that facilitates the proper choice of nucleotides provides robustness to this noise, which, at the same time, prevents robustness against mutations caused by DNA replication. In other words, mutational robustness and robustness to noise are not only associated here, they are one and the same.

### Other Examples

In making the case for an association between mutational robustness and robustness to nongenetic change, I have thus far focused on biological systems from previous chapters. However, other studies hint at the same principle in a variety of systems. One such study examined important life history characters such as fecundity and lifespan in fruit flies. It found that characters more robust to genetic change in the form of transposon-induced mutations are also more robust to temperature variation in the environment (520).

Another example regards bacterial chemotaxis (14, 31, 38, 62, 524). Bacteria such as *E. coli* can approach food sources by detecting a gradient of a chemical compound and swim upstream against the gradient. The necessary changes of swimming direction involve changes in the rotation of a cell's flagellae: When its flagellae rotate in one direction, the cell swims in a straight line; when they rotate in the other direction, the cell tumbles erratically and thus reorients itself. Through a combination

of straight swimming and random reorientation, a cell eventually reaches the source of the chemoattractant. This process works for chemoattractant concentrations varying over several orders of magnitude. It is also highly insensitive to the slope of a concentration gradient.

If a cell is exposed to a constant, nongraded concentration of chemoattractant, its flagella switch randomly and at a constant frequency between rotational states (straight-swimming and tumbling). When the chemoattractant concentration in the surrounding medium increases—not in a graded fashion, but uniformly everywhere in the medium—the frequency of rotational switching first changes, favoring directional swimming, and then reverts back to the original frequency. This process of *adaptation* to concentration changes is critical for chemotaxis. *E. coli* mutants defective in it are also defective in chemotaxis. The insensitivity of chemotaxis to absolute chemoattractant concentrations also derives from this adaptation ability.

The biochemical machinery allowing chemotaxis involves interactions among a variety of proteins (62, 524). Briefly, chemoattractant receptors detect changes in chemoattractant concentration, which causes phosphorylation of signaling proteins that change the motor driving flagellar rotation. Adaptation to changed chemoattractant concentrations is mediated by methylation of the receptor proteins. Relevant to my argument is that the precision of adaptation to changed chemoattractant concentration is highly robust to changes in the concentration of the signaling proteins. Specifically, what is robust is the fraction of time a cell's flagellae spend in either rotational state after having adapted to a changed chemoattractant concentration: The concentration of individual signaling proteins can vary more than 100-fold, yet adaptation remains perfect (14). The source of such variation is again irrelevant: Whether it is caused by intracellular noise or by mutations changing gene expression, adaptation and chemotaxis will continue to occur properly.

In sum, available evidence suggests that robustness to mutations is generally associated with robustness to nongenetic change. It takes little thought to appreciate that exceptions to this rule would be highly surprising. The reason is that robustness means insensitivity to changes in a system's composition, and the cause of such change—genetic or not—is irrelevant.

### Natural Selection Can Modify Robustness to Nongenetic Change

The previous sections showed that all organisms are subject to nongenetic change, and that robustness to such change is accompanied by mutational robustness. Thus, two of three conditions necessary to explain mutational robustness as a by-product of robustness to nongenetic change

are met. What remains to be shown is that robustness to noise can change in evolution, and that this change can occur under less restrictive conditions than the evolution of robustness in response to mutations. The most important question in this regard is whether genes influence robustness to nongenetic change. The reason is, again, that natural selection requires heritable genetic variation, variation in robustness to nongenetic change.

Consider environmental change first. That an organism's response and sensitivity to environmental change depends on its genotype has been known for a long time and is uncontroversial (59, 99, 109, 349, 381, 383–385, 402, 542, 565, 567, 614). In quantitative genetics, this dependency is known as a genotype-by-environment interaction (349). Many and diverse traits—wing coloration of butterflies, thorax length of fruit flies, seed production of annual plants, variation in leaf shape of water plants—vary genetically in their response to environmental change, either within populations, among populations, or among species (59, 99, 109, 381, 383–385, 402, 542, 565, 567, 614). Laboratory evolution experiments can even modify such robustness. For instance, Scheiner and Lyman evolved fruit flies whose thorax size was more or less sensitive to temperature variations, compared to an ancestral population (486).

The effects of internal noise on organisms are similarly influenced by genes. Early indications came from a phenomenon that was studied long before the ubiquity of gene expression noise was appreciated. This phenomenon is fluctuating asymmetry, variation within an organism in paired bilaterally symmetric traits. Examples of such traits include left and right vertebrate limbs, left and right wing feathers of birds, and various skull features of vertebrates, such as corresponding left and right teeth (358, 551, 557). Most such bilaterally symmetric traits show differences within one organism. Because the genes guiding the development of symmetric structures are identical within the organism, the structures should be identical if no random variation—noise—occurred during their embryonic development. However, fluctuating asymmetry is ubiquitous, which attests to the importance of noise. Fluctuating asymmetry is genetically controlled, because asymmetry often increases when genetic variation is lost, such as after inbreeding (358). Also, fluctuating asymmetry can increase in hybrid organisms whose parents come from different populations (358).

Some experimental work on gene expression noise I discussed above also shows that genes can influence such noise. For example, in a study of gene expression in the bacterium *E. coli*, some bacterial strains exhibited twice as much noise in gene expression than others. This is due to variation in the *recA* gene, a recombination and DNA repair gene. Cells defective in *recA* exhibit greater noise in gene expression than other cells (138). *RecA*'s function in rescuing stalled DNA replication processes may

be responsible for this phenomenon: *recA* defective cells may show temporary differences in gene copy numbers within cells (138). Such differences would clearly lead to increased variation in gene expression within and among cells. A second example comes from the expression of green fluorescent protein in the bacterium *Bacillus subtilis* (425). Mutations affecting the translational efficiency of its messenger RNA, such as mutations altering the messenger RNA's ribosome binding site, can greatly influence gene expression noise. Specifically, genes encoding green fluorescent protein whose messenger RNAs can be translated more efficiently show greater expression noise. The reason is that efficient translation effectively amplifies the variation in messenger RNA copy number that is caused by transcriptional noise (425). A final example comes from a study of developmental genes in the fruit fly *Drosophila* which showed that the expression pattern of the *hunchback* gene varies much less across fly embryos than the expression pattern of other genes such as *bicoid*. This robustness of *hunchback*'s expression can be disrupted by mutations in the gene *staufen* (243), which shows that it is genetically controlled.

In sum, there is no doubt that robustness to nongenetic change varies genetically. The most important condition for the evolution of robustness to noise is thus met. But can selection modify robustness to nongenetic change more readily than robustness to mutations? To see why this must be the case, recall from the previous chapter that selection for mutational robustness occurs only if a population contains variation for mutational robustness and if mutations occur in the systems whose robustness differs. Mutations, however, are rare events occurring at a typical rate of  $10^{-6}$  per gene and generation. In contrast, environmental change and internal noise are much more frequent sources of variation. Every biological system is subject to them during every moment of its existence.

In a study pertaining to this issue, Michael Lynch estimated the amount of variation introduced into phenotypic characters through mutations ( $V_m$ ), relative to the amount of variation introduced through nongenetic factors ( $V_e$ ) (347). More precisely,  $V_m$  and  $V_e$  are the contributions newly arisen mutations ( $V_m$ ) and nongenetic influences ( $V_e$ ) make to the variance of a phenotypic character among individuals in a population. Lynch's study covered a broad range of characters and organisms, including biomass of barley, bristle numbers and viability in fruit flies, pupal weight in flour beetles, and skeletal traits such as ulna length in mice. He found that the ratio  $V_m/V_e$  varies between  $10^{-2}$  and  $10^{-4}$ , depending on the trait. This shows that  $V_m$  is always much smaller than  $V_e$ , underscoring the point that mutations are a minor source of variation compared to the environment. Put differently, the mutations that occur every generation in a population account for no more than 1% of the variation in phenotypic characters.

Thus, if robustness to nongenetic influence is advantageous, this advantage will cause much greater variation in fitness than mutational robustness. There is no need to invoke special mechanisms such as second-order-selection, as was necessary for mutational robustness. Except where sensitivity to noise is advantageous (chapter 18), systems more robust to nongenetic change simply confer higher fitness. Textbook population genetic theory thus suffices to explain the evolution of their robustness. Not surprisingly, population genetic models predict that evolution of robustness to nongenetic change is easily accomplished (182, 448, 591).

### The Smoking Gun?

All this shows that robustness to nongenetic change can evolve. But did it? That is, did robustness to nongenetic change in many systems evolve as an adaptation to such change? Some of the systems I discussed suggest so. For example, recall that the robustness of the genetic code (chapter 3) reflects the experimentally observed distribution of translation errors among codon positions. Specifically, the rate of translation errors is much greater at the third position of a codon than at the first position, whose error rate is in turn greater than that at the second position. Correspondingly, code robustness at each of the codon positions mirrors exactly the observed pattern of translational errors. That is, robustness of the third codon position is greater than at the second position, which is in turn greater than at the first position. We have no evidence that mutation rates vary among codon positions in this manner, so this pattern is a likely signature of evolved robustness for translation errors (171).

Unfortunately, analogous and unequivocal signatures of evolved robustness are not known for most systems robust to noise. This holds even for the robustness or plasticity of macroscopic traits to environmental change, which has been under scrutiny since the early 20th century. How selection shapes their robustness is still controversial (485, 486, 567). The same holds even for molecular networks whose gene interactions are well understood, such as that responsible for chemotaxis. Their robustness to noise—and hence to mutations—smacks of an adaptation to noise, and it would be surprising if this turned out not to be the case. However, rigorous proof is another matter, and a subject for future research.

# 18

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## Robustness and Fragility: Advantages to Variation and Trade-offs

Robustness in living systems deserves explanation, because such systems are complex and one might thus expect them to be fragile, much like a house of cards. In contrast, we saw that most living systems can tolerate much mutational pressure, and often to an astonishing extent. However, there are notable exceptions. Some systems inside organisms can be exceedingly fragile, in the sense that their properties vary extensively in response to mutations. Although some such fragility may be a simple by-product of complexity, fragility may also have two other causes. First, it can be advantageous. Second, even where fragility is disadvantageous, trade-offs between robustness and other aspects of system function may exist, trade-offs that can prevent the increase of robustness in evolution. For example, maximally robust enzymes would not be able to catalyze chemical reactions, because the molecular motions underlying catalysis are favored when thermodynamic stability and robustness are limited. Fragility in both of these cases results from evolution by natural selection, which is why I explore them further here.

### Advantageous Variation

Robustness reduces the expression of genetic variation as phenotypic variation. It can thus reduce natural selection's ability to modify organismal features. This can be beneficial if a feature already serves a function well. In the lingo of population genetics, such a feature is under stabilizing selection. That is, mutations affecting the feature confer inferior fitness to the organism and selection will eliminate them. Because most organismal characters are probably under stabilizing selection most of the time (80), robustness is beneficial most of the time. However, another mode of selection, directional selection, can be of great importance as well. Directional selection means that room for improvement by natural selection exists, and that mutations can generate variants with superior fitness. Such improvement will be most rapid if much phenotypic variation

with a genetic basis exists. Thus, where directional selection rules, it is desirable to maximize heritable phenotypic variation and thus a trait's variability—and, in this sense, its fragility in response to mutations.

## Two Ways to Increase Variation: More Mutations or Less Robustness

One of two ways to increase phenotypic variation is to increase genetic variation. To do so requires an increase in the rate at which mutations occur, because mutations are the root cause of all genetic variation. The most prominent examples of beneficial increases in mutation rates come from bacterial colonies that are subject to environmental stress, especially the stress of starvation (48, 167, 328, 340, 505, 506, 538, 543). Such cells can drastically increase their mutation rates. For example, a study of 787 natural isolates of the bacterium *Escherichia coli* showed that 40% of these isolates increased their mutation rate tenfold in old, nutrient-deprived colonies. Thirteen percent increased the mutation rate more than a 100-fold (48). This mutation rate increase, which disappears when environmental conditions improve, is under genetic control. For example, mutations of the bacterial transcription factor  $\sigma^S$ , which is responsible for the expression of stress-responsive genes, can abolish the mutation rate increase. Several biochemical mechanisms can account for a transient increase in mutation rate (167). They include increased expression of DNA polymerases prone to make replication errors, and a reduction in proteins such as MutS, which is responsible for repairing mismatched nucleotides in double-stranded DNA (48).

Under threat of starvation, an increased mutation rate provides obvious advantages. While most of a colony's individuals might perish from the resulting deleterious mutations—or eventual starvation—a few mutants may arise that can escape the stressful situation, such as through their ability to metabolize a previously useless food source. However, it is difficult to prove conclusively that the increase in mutation rate is an evolved adaptation to the need for more genetic variation in stressful times. The reason is that under starvation, cells may reduce DNA repair because it is energetically costly, and thus reduces their short-term survival (505). If so, their increased (and beneficial) mutation rate would be a by-product of an energy conservation mechanism.

Another case of potentially advantageous variation occurs in immune systems. The textbook example is the adaptive immune system of vertebrates. Here, genes undergo extensive recombination and hypermutation to produce a maximally diverse set of antibodies. It is difficult to subsume this example under the robustness–fragility dichotomy, because

here variation itself—in antibody shapes—is the valued trait. Nonetheless, this is clearly another case where constancy of a feature is disadvantageous.

A variation of this theme is found in some of the infectious agents that immune systems combat: pathogenic bacteria. Such bacteria express surface proteins necessary for host infection. Unfortunately for the pathogen, these surface proteins can also serve as antigens that trigger the host's immune response. Bacteria have found a way to escape this dilemma: Different cells in one bacterial population express different surface antigens, or some cells may not express any of these antigens at all (454, 479, 620). Doing so allows part of a population to survive if the host can recognize their surface proteins. Conversely, it allows another part of the population to infect if the host is susceptible. The mechanisms generating such variation differ among bacterial species. They include changes in the orientation of promoters necessary for gene expression, or gene conversion, where the sequence of one expressed gene is altered through recombination with other, silent copies of the gene.

The above examples illustrate various mechanisms to increase variation available to natural selection by increasing mutation rates, either across a genome or for individual genes. A second potential mechanism to increase variation leaves the mutation rate untouched. Instead, it increases fragility of biological systems to new mutations or to existing variation—the result of past mutations. This phenomenon has also been called anti-robustness, anti-redundancy, or decanalization (104, 306, 462). There is currently only very limited empirical evidence for it. This evidence regards the usage of codons in proteins of two important human pathogens and rests on the following observations (444a). Two codons encoding the same amino acid may differ in the likelihood that a random nucleotide change at one of the codon positions causes a change in the encoded amino acid. For example, the codon CGA encodes the amino acid arginine. It has eight one-mutant neighbors different from stop-codons, that is, codons that differ from it at one nucleotide position. Four of these one-mutant neighbors also encode arginine. Thus, a fraction 4/8 of missense mutations in this codon would have no effect on the encoded amino acid. In contrast, the codon AGA also encodes arginine, but only two of its one-mutant neighbors also encode arginine. One might thus call the codon AGA less robust to point mutations, because 6 out of 8 mutations in it lead to a change in the encoded protein's amino acid composition, as opposed to the codon CGA, where only 4 out of 8 mutations cause such change. Surface proteins of the two human pathogens *Mycobacterium tuberculosis* and *Plasmodium falciparum* that are likely targets of the host immune system preferentially contain codons of low robustness, that is, codons whose mutation is likely to lead to amino acid changes (444a). This suggests that such proteins have been under past

selection favoring amino acid variation in response to mutation. This variation may help the pathogen evade the host's immune system. Note that this mechanism to increase variation is different from that in the surface antigens I mentioned above: Not the mutation rate is increased, but the amount of variation produced in response to a given number of mutations.

Increases in variability have also been proposed to occur in the wide spectrum of developmental pathways influenced by the molecular chaperone Hsp90 (471). As I discussed in chapter 11, Hsp90 normally buffers much genetic variation in development. However, under extreme environmental stress, misfolded proteins accumulate and most of a cell's chaperones may be needed to refold these proteins. The remainder may not be sufficient to buffer all genetic variation, and more of this variation thus becomes visible on the phenotypic level. Most such variation would be deleterious, but some of it may be beneficial. Thus, it has been argued, Hsp90 may act as a capacitor of genetic variation, releasing variation in times of need, which would have effects similar to an increase in the mutation rate (471). This phenomenon would be of special interest if it had evolved specifically to provide needed variation in stressful conditions. One major problem with this hypothesis (for another one see below) is that Hsp90 has an obvious primary function: it provides robustness against thermal noise. This means that the capacitance of Hsp90 may be an accidental by-product of its primary function.

Aside from the above example on codon usage, we currently thus have no solid evidence of an increase in the fragility of any trait—either on an ecological or an evolutionary timescale—that has evolved for the purpose of generating increased variation. To identify more such traits would be a very worthwhile subject of further study. However, the following arguments suggest that evolution of such increased fragility may occur only under a limited range of conditions.

### Mechanisms Increasing Variation Cannot Evolve Through Individual-Based Selection

Individual-based selection—the best-corroborated kind of natural selection—changes a population's composition by selectively eliminating individuals in it. Other kinds of natural selection act on higher levels of biological organization, such as groups of individuals with specific properties (507). Note that the conception of both individual and group here depends on one's perspective. An individual might be a many-celled organism, a single-celled organism, or a cell that is part of a cell lineage inside a many-celled organism. A group might be a collection of genetically unrelated organisms; alternatively, it might consist of genetically related

organisms, such as in kin selection, where closely related individuals can evolve features disadvantageous to the individual but advantageous to its relatives (220). That is, a feature costly to one individual in terms of survival and reproduction can become established if it aids this individual's relatives, because it can be propagated through these relatives. The more closely related two organisms are, the easier it is for kin selection to evolve traits disadvantageous to the individual. Arguably, kin selection would be most effective in asexually reproducing organisms or cells, because here organisms in the population are much more closely related than even the closest relatives in sexually reproducing organisms. Specifically, any two organisms in an asexual population share nearly 100% of their genes, as opposed to even parents and their offspring in most sexually reproducing populations, which share, on average, only 50% of their genes.

Mechanisms to blindly increase variation through an increase in mutation rates generally do not provide an advantage to an individual. The reason is that the vast majority of random mutations are deleterious, thus decreasing any one individual's fitness. However, mutations may provide an advantage to a group, because they increase the likelihood that *some* individual can overcome whatever limits the population. In other words, the evolution of mechanisms to increase genetic variation will usually require selection on a level higher than the individual. The microbes whose increased mutation rates I discussed above are among the best candidate organisms in which such higher level selection might be effective. First, they are microparasites, among which group selection is well documented (507). Second, many such microbes reproduce asexually. Traits that are disadvantageous to the individual but potentially advantageous to all its relatives—the entire population—can readily evolve under these circumstances.

The only major exception to the rule that individual-based selection is insufficient for the evolution of increased mutation rates holds for organisms where recombination rates are very low, among them many bacteria (505). Specifically, if recombination rates are very low, genes conferring high mutation rates—mutator genes—can temporarily increase in frequency by means of individual selection. Here is how this can occur. Occasionally, the mutations triggered by a mutator gene may include an advantageous mutation in a gene near the mutator gene. In the absence of recombination, this advantageous mutation will remain linked indefinitely to the mutator gene. If selection strongly favors the advantageous gene, its frequency in the population will rise, and so will that of the mutator gene. Importantly, this “hitchhiking” mechanism works only in the absence of recombination (505). Arguably, here the mutation rate increases in evolution not because it confers an advantage, but—despite its disadvantage—as a by-product of selection favoring one advantageous mutation.

Similar to mechanisms that increase variation, mechanisms that decrease robustness to existing variation also provide a serious disadvantage for the individual. The reason is the same: Most variation is deleterious to the individual. This observation makes it even more problematic to explain the capacitance of molecular chaperones as an evolved mechanism to increase variation. In organisms like fruit flies, where this role of chaperones has first been characterized, the potential for group or kin selection that allows “self-sacrificial” traits to evolve is much weaker than in microbial parasites. The reason is that the average genetic relatedness among individuals in an outbred, sexually reproducing population is low. In addition, high recombination rates in such organisms would prevent the evolution of alleles decreasing robustness through the individual-based mechanism I just outlined.

Krakauer and Plotkin (306, 308) suggest that a hypothetical decrease in robustness may sometimes serve a purpose different from that of increasing variation. Among their examples is the role of tumor suppressor genes such as the human gene *p53* in programmed cell death or apoptosis. Its product responds to some (nonlethal) mutations by arresting the cell division cycle and by causing death in the affected cells. This can be viewed as a mechanism for increasing fragility of cells to mutations. Without this mechanism, mutations could accumulate that lead to uncontrolled cell proliferation and cancer. In other words, Krakauer and Plotkin hypothesize that fragility can serve to purge mutations from a population of cells or organisms, in order to serve a larger whole—a many-celled organism or a population. In large populations, natural selection is especially effective at purging such variation, because it is a stronger evolutionary force than random genetic drift. In support of this notion, the mean fitness of large populations may increase with increases in such fragility (306).

Again, it is important to be aware that such a mechanism can generally not evolve through individual-based selection among cells or organisms, because it provides a disadvantage for the individual cell. However, in a clonally related cell lineage within an organism, the main condition for the evolution of self-sacrificial behavior—close genetical relatedness of the lineage’s members—may well be met. Despite the above limitations, the possibility that fragility has evolved to purge mutations is intriguing and worth exploring further, although we currently have no incontrovertible evidence in favor of it.

### Trade-offs

The above examples illustrate two potential causes of increased genetic variation in a population, increased mutation rates and decreased robustness.

Because neither of these causes provides an advantage to the individual, they are likely to evolve only under limited circumstances. In contrast, another evolutionary cause of limited robustness may be much more widespread. For illustration, I discuss this phenomenon in the context of a specific example: overlapping genes (304). Two genes overlap if either their transcribed regions on DNA overlap or their translated regions on messenger RNA overlap. Overlapping genes are abundant in bacteriophages, viruses, bacteria, and mitochondria (403). Such overlap can include all of a gene's coding region and it can involve more than two genes. One example is the DNA polymerase gene of the hepatitis B virus. Not only do the ends of this gene overlap with two other viral genes, the gene also completely contains a third gene responsible for expressing viral surface proteins (374). Overall, more than 60% of the DNA polymerase gene is also part of another gene.

Gene overlap has many potential benefits. They include increased replication speed through smaller genome size, easier packaging of genetic material into viral particles, and co-regulated gene expression to ensure proper amounts of gene product (304, 403). These benefits are especially important in viruses, which produce huge numbers of offspring in small amounts of time. For instance, even very small differences in genome replication rates can have large effects on viral fitness. However, gene overlap also has a potential disadvantage, a disadvantage that may weigh heavily in some viruses with mutation rates a million times higher than those of eukaryotic genomes (123). That is, gene overlap may increase the fragility of a viral population to mutations. Even though gene overlap reduces the number of nucleotides in two genes, and thus reduces the rate at which mutations affect two genes, any one mutation can have a much more severe effect than if two genes do not overlap. The reason is that any one mutation may now impair two gene products. Empirical evidence supports this notion. For example, in regions of gene overlap in the hepatitis B virus genome, nucleotide substitutions that do not cause an amino acid change in one of the gene products (but that may cause such a change in the other gene product) occur at a rate five times lower than in regions without overlap (377). This means that during the evolution of hepatitis B virus, many more mutations were eliminated from regions of gene overlap than from other regions. The reduction of robustness caused by overlap depends on many details, including the extent of overlap, the orientation of the overlapping genes, and the relative position of their reading frames (304).

As these observations show, gene overlap provides a simple example of a trade-off between features such as increased replication rate, on one hand, and robustness, on the other hand. Thus far, I have always treated robustness as an isolated feature of a system, a feature that can be treated independently from other features. Although doing so facilitates understanding

the causes of robustness, it does not reflect biological reality. Trade-offs like this one can limit evolutionary increases in robustness.

I now suggest a simple framework to make precise the notion of trade-offs between robustness and other traits, especially components of fitness. The most important question such a framework must address is this: Can robustness increase in the face of trade-offs, or will—in terms of the above example—the virus with increased replication rate always win. If so, trade-offs would inevitably result in fragility. Although the framework has limitations—for one thing, it ignores the complications of sex—its applicability goes far beyond the merely illustrative example of overlapping genes. It applies to any quantitative trait and any quantitative measure of robustness. In addition, it applies to robustness against mutations and against noise alike—because both of them are linked—with the usual note of caution that selection for mutational robustness is much weaker than selection for robustness to noise.

We can view an organism's ability to survive and reproduce as some function  $w$  of two (or more) traits, one of which is the robustness  $r$  of some system inside the organism, and the other one is some feature  $f$ , such as genome replication speed in the above example:

$$w = w(r, f)$$

Both of these traits influence  $w$  positively, that is, increasing one of them leads to an increase in the ability to survive and reproduce. Cast in mathematical terms, this means that both the partial derivatives  $\partial w / \partial r$  and  $\partial w / \partial f$  are greater than zero.

The scenario important here is one where these traits cannot vary independently from one another. (If they could vary independently, natural selection could mold them independently, and no trade-off exists.) In the case of a trade-off, an increase in robustness  $r$  causes a reduction in  $f$  and vice versa. How to best define a trade-off depends on how  $r$  and  $f$  depend on each other. There are two principal possibilities.

First, both robustness  $r$  and the other feature  $f$  may depend on the same underlying variable  $v$ . For instance, the extent  $v$  to which two genes overlap influences both robustness  $r$  and replication speed  $f$ . In this case, we have

$$w = w[r(v), f(v)] \quad (18.1a)$$

A trade-off then requires that a change in  $v$  (overlap) has opposite effects on  $r$  (robustness) and  $f$  (replication speed). Mathematically speaking

$$\frac{dr}{dv} \frac{df}{dv} < 0 \quad (18.1b)$$

Most importantly now, a mutation that increases robustness can increase its frequency in a population only if this mutation also causes a net increase in the ability to survive and reproduce. In other words, if an increase in  $v$  increases robustness, then it must also increase  $w$ . If an increase in  $v$  decreases robustness, then it must also decrease  $w$ . Mathematically speaking, robustness of a system can thus increase only if

$$\frac{dw}{dv} \frac{dr}{dv} > 0 \quad (18.1c)$$

The second possibility for the relation between  $f$  and  $r$  is that one can be written as a function of the other. For example, if  $f = f(r)$ , then one can write

$$w = w[r, f(r)] \quad (18.2a)$$

A trade-off then implies that

$$\frac{df}{dr} < 0 \quad (18.2b)$$

Again, robustness can increase in evolution only if an increase in robustness entails a net increase in the ability to survive and reproduce, that is, if  $dw/dr > 0$ . Because  $w$  depends on both  $r$  and  $f$ , it is necessary to apply the chain rule of differentiation to obtain the following criterion:

$$\frac{dw}{dr} = \frac{\partial w}{\partial f} \frac{df}{dr} + \frac{\partial w}{\partial r} \frac{dr}{dr} > 0$$

As above,  $\partial$  indicates partial differentiation of a function of two variables, whereas  $d$  indicates differentiation of a function of one variable. The above relation shows that robustness can increase as long as

$$\frac{df}{dr} > -\frac{\partial w/\partial r}{\partial w/\partial f} \quad (18.2c)$$

A completely analogous relation,  $dr/df > -(\partial w/\partial f)/(\partial w/\partial r)$ , can be derived in the converse case, where  $r$  can be written as a function of  $f$ .

In sum, depending on how the relation between robustness and other traits is defined, (18.1c) or (18.2c) states necessary conditions for an increase in robustness in the face of a trade-off.

With this notation in hand, I can now revisit the question of whether robustness can evolve in the face of trade-offs. I revisit this question for

the example of overlapping genes and make a simple observation: This question has no general answer even where  $f$  and  $r$  are related in a very simple way. I emphasize that the following is a mere caricature of the complexity of the problem, intended for illustration only. More sophisticated representations are conceivable (304), but their conclusions are equally limited if empirical data is in short supply.

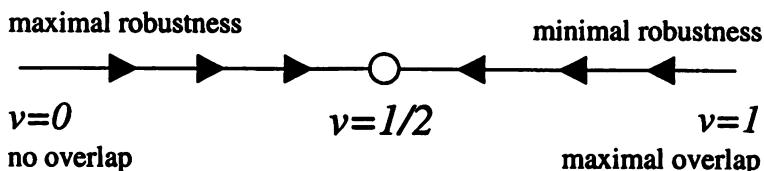
Assume that the trait  $f$  in competition with robustness  $r$  is replication speed. Assume further that both robustness and replication speed depend linearly on the overlap  $v$  between two genes. If we scale  $v$  such that it ranges from  $v = 0$  (no overlap) to  $v = 1$  (one gene contained within the other), then replication speed can be written as  $f(v) = f_- + v(f_+ - f_-)$ . Here,  $f_-$  and  $f_+$  correspond to the minimal replication rate, realized without overlap ( $v = 0$ ), and the maximal replication rate, realized with complete overlap ( $v = 1$ ). The fraction  $r$  of a population of viruses that suffers no deleterious mutations varies between some small value  $n_-$  (complete overlap) and some larger value  $n_+$  (no overlap). It is a measure of robustness and can be written as a function of overlap,  $r(v) = n_+ - v(n_+ - n_-)$ . The reproductive output or growth rate  $w$  of a viral strain with overlap  $v$  among two of its genes is proportional to the product of its replication speed and the fraction of the population that does not suffer deleterious mutations. In other words, we can write  $w[r(v), f(v)] \propto r(v) f(v)$ . Thus, we have an instance of our first case above, where both robustness and replication speed depend on one common variable  $v$ . It is easy to verify that an increase in either  $r$  or  $f$  increases  $w$ , and that the criterion (18.1b) for a trade-off is met. Under what conditions can robustness increase in evolution? We need to apply criterion (18.1c). A little algebra shows that

$$\frac{dw}{dv} \frac{dr}{dv} > 0 \Leftrightarrow v > \frac{f_+ n_+ + f_- n_- - 2f_- n_+}{2(f_+ - f_-)(n_+ - n_-)} \quad (18.3)$$

This is a complex relationship depending on parameters about which we have no empirical information. Only in some special cases will it reduce to a simple expression. For example, if complete overlap doubles the replication speed ( $2f_- = f_+$ ), and if it halves the likelihood that no deleterious mutations occur ( $2n_- = n_+$ ), the above relation reduces to

$$\frac{dw}{dv} \frac{dr}{dv} > 0 \Leftrightarrow v > \frac{1}{2} \quad (18.4)$$

Expressed in words, mutations that increase robustness by decreasing overlap have a net advantage and become established if two equally long genes overlap by more than 50% of their length. Conversely, mutations that decrease robustness and thus increase overlap can increase in frequency



**Figure 18.1** The evolution of gene overlap and robustness as a result of the trade-off between replication speed and neutral mutation rate for a hypothetical example of overlapping genes. The arrows indicate that at high robustness (low overlap,  $v < 1/2$ ), mutations that decrease robustness are favored, whereas at low robustness (large overlap,  $v > 1/2$ ) mutations that increase robustness are favored.

if two genes overlap by less than 50%. Put differently, robustness provides a net disadvantage when high and a net advantage when low. The result of the trade-off in this case is an evolutionarily stable overlap of 50% implying half-maximal robustness (Figure 18.1).

While theoretical analysis using the above framework is straightforward, we have no quantitative data on the relationship between gene overlap, robustness, and any of the possible advantages of overlap. We can thus not go beyond this theoretical analysis. Unfortunately, the same holds for other trade-offs. We do know that they exist but we have no data quantifying them. An especially prominent example is that of the structural robustness of RNA and proteins (chapters 4 and 5). Maximal robustness may allow a protein to adopt its native conformation rapidly and to attain the same conformation even when subject to mutations or elevated temperature. However, the function of some proteins—especially enzymes—relies on subtle conformational changes, changes that occur more slowly if a structure is highly robust and thermodynamically stable. This trade-off between robustness and conformational motion is likely to favor intermediate rather than maximal robustness. Similarly, RNA molecules that adopt the same most stable secondary structure can differ greatly in mutational robustness and thermodynamic stability of this structure. If a structure best serves a biological function, then the most robust molecule folding into the structure should clearly be favorable. However, robustness of this structure can have adverse effects. For example, RNA secondary structures necessary for replication or translation of viral genomes must have limited thermodynamic stability: They must be able to unfold to let the translation or replication machinery read the RNA's nucleotide sequence. Because thermodynamically stable sequences are also mutationally robust, this means that maximal mutational robustness may be unfavorable. Again, the trade-off between robustness and the ability to unfold will prevent the evolution of maximal robustness. While this trade-off can be described mathematically for model sequences

and structures (609), relevant empirical data are absent for actual RNA molecules.

Our information on the functions of many biological systems—from molecules to networks—is generally fragmentary. All we know is that many systems carry out several functions inside the organism. One might thus suspect that such systems are so highly constrained by their functions that the evolution of robustness—whether to mutations or to noise—may be a negligible factor in their evolution. That is, if robustness faces trade-offs not with one trait  $f$  but with many traits  $f_1, \dots, f_n$ , there may be only one system configuration that fulfills all functions optimally. Proteins are good candidates for such highly constrained systems, because we know that many proteins are multifunctional. However, empirical data on protein evolution suggests that such severe constraints are the exception rather than the rule. That is, for most proteins there are many equivalent ways—a vast neutral space—to fulfill the constraints the protein has to meet. This is best illustrated by studies of protein evolution, studies that compare proteins with similar functions in many different organisms. Such studies, of which I presented a smattering in chapter 5, show that highly constrained proteins are not frequent. Many proteins can retain similar functions yet diverge dramatically in their amino acid sequences.

Despite such qualitative insights, which show that there is room for the evolution of robustness, we sorely lack quantitative, empirical information on the trade-offs between robustness and other traits of biological systems. Ultimately, we will understand why some systems are more robust than others only when such information becomes available.

### The Opposite of Trade-Offs: Robustness for Free

I have so far emphasized trade-offs between robustness and other traits. However, examples for the opposite exist as well. That is, robustness and other traits can act synergistically to increase reproduction and survival. Put differently, a trait that increases survival or reproduction can also increase robustness. Robustness does not have to be costly. (In terms of the above mathematical framework, such synergism means that  $(dr/dv)(df/dv) > 0$  or that  $df/dr > 0$ , depending how the relation between traits is best represented.)

The clearest examples of a synergism between robustness and other traits come from metabolism. Take a metabolic pathway under selection to maximize production of the pathway's end product, and consider genetic variation at one of its enzyme-coding genes, variation that affects enzyme activity. Natural selection will drive alleles that increase enzyme activity to fixation. However, doing so will also increase the robustness

of the pathway, both to mutations reducing enzyme activity and to fluctuations in enzyme concentrations due to noise. The reason is (chapter 8) that flux through a metabolic pathway shows a diminishing-return relation with enzyme activity. That is, if enzyme activity is low, a small increase in enzyme activity leads to a larger increase in flux than if enzyme activity is high. The same holds for mutations or noise that reduce enzyme activity. That is, a given amount of (mutational) change in enzyme activity has smaller effects on flux at lower enzyme activities than at high activities. Thus, both flux  $f$  through a metabolic pathway and robustness  $r$  of a metabolic pathway increase with the same variable  $v$ , which is enzyme activity. Molecular chaperones provide a similar example. Their primary function is to protect proteins against misfolding, which also increases robustness to both mutations and thermal noise.

Where robustness comes for free, the question arises as to whether its evolution should at all be viewed as an adaptation to mutations or to noise. For robustness to noise, the answer may depend on the trait, but for mutational robustness, the answer is no. To stay with the example of metabolism, selection to increase enzyme activity through increased metabolic flux is highly effective and it can change flux within a few generations, whereas selection favoring robustness to mutations in enzyme-coding genes is less effective and operates on much slower timescales—if at all—because mutations are so rare. Mutational robustness in metabolic pathways is thus best viewed as a by-product for selection on increased enzyme activity.

In sum, a lack of robustness in a biological system may sometimes be a by-product of a system's complexity, or of the fact that the system embodies a rare solution to a biological problem (chapter 13). However, where natural selection can influence a system's robustness, two additional explanations for a lack of robustness are possible. First, natural selection may favor increased variability in some systems. In other systems, trade-offs with other traits may prevent an evolutionary increase in robustness. We have no quantitative and only limited qualitative information about these two mechanisms. For instance, the examples I just discussed—molecular structures and metabolic flux—are among the very few where we know about trade-offs and synergisms between robustness and other important traits. The rest of the systems in previous chapters of this book are uncharted territory. I could speculate endlessly whether the robustness of the genetic code, the redundancy provided by gene duplications, gene interaction networks, or organismal development is costly, advantageous, or perhaps does not affect any other traits. However, this is ultimately yet another wide-open area for empirical research.



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## **Part IV**

### **ROBUSTNESS BEYOND THE ORGANISM**



## Robustness in Natural Systems and Self-Organization

In this chapter, I point out parallels and, more importantly, differences between robustness in living systems—organisms and their parts—and other, nonliving systems. One important commonality between robustness in living and other systems is obvious: Something—either the state of a system or the system itself—persists in the face of perturbations. However, at least two differences are more significant than this commonality. First, only organisms have genetic material and can thus be robust to mutations in this material. Second, and more importantly, most nonliving systems are not subject to the force of natural selection, which may favor robust living systems. The robustness of nonliving systems must have a different origin, an origin that can be subsumed in one word: self-organization.

Systems robust to perturbations outside organismal biology are so widespread that it would be hopeless to strive for representative examples covering most fields of science. The relevant literature in nonlinear dynamics (238), chemical pattern formation (392), engineering (327), ecology (212, 331), and the social sciences is vast, too vast to do it justice in a single book, let alone a single chapter. To make matter worse, different fields use the word robustness in different connotations; and they use different words to describe robustness in the sense I use it here. These factors make it difficult to discuss robustness exhaustively. I will illustrate the above differences and the role of self-organization in robustness of nonliving systems with merely two select examples. The choice of these examples is arbitrary except that they serve to make the key point. Also, for maximum contrast I chose maximally different kinds of systems: One of them is a system whose parts are molecules, and the other is a system whose parts are whole organisms. (Such systems, ecological communities, are not themselves alive, although their parts are.)

## A Trivial Example

Consider the following stoichiometric equation describing a chemical reaction:



In this reaction, one molecule of substance *A* reacts at a rate  $k_+$  with one molecule of *B* to form two molecules of *B*. The reaction is reversible, and thus two molecules of *B* also react, at rate  $k_-$ , to form one molecule of *A* and *B*. Both the forward and the backward reaction are autocatalytic, in the sense that the more *B* is produced, the faster they proceed. Such autocatalytic reactions occur both in inorganic and organic chemistry, for instance in the oxidation of hydrocarbons and in oxidations involving nitric acid (69).

According to the law of mass action, the rate at which a chemical reaction proceeds is proportional to the products of the reactant concentrations. If the concentration of *A* is held constant, then one can describe the change in the concentration *b* of *B* with one simple differential equation:

$$\frac{db}{dt} = k_+ab - k_-b^2 \quad (19.2)$$

Here *a* is the (constant) concentration of *A*. Eventually, the reaction (19.1) as described by (19.2) reaches a dynamic equilibrium at which the concentration of *b* does not change, namely

$$b = \frac{k_+a}{k_-} \quad (19.3)$$

This equilibrium is stable in the following sense. If the concentration of *B* rises above it, the reverse reaction will reduce its concentration, and if the concentration falls below it, the forward reaction will increase its concentration. The equilibrium is dynamic in that many molecules of *B* may be generated and destroyed at any moment, even though the net rate of change in *b* is zero. One could say that the reaction (19.1) has an equilibrium that is “robust” to changes in the concentration of *B*: No matter how *b* changes, the equilibrium will eventually be restored.

Most will find this example of “robustness” hardly exciting. It is akin to that of marbles in a bowl, which return to the lowest point after being stirred up. Similar stable equilibria occur in many unrelated systems, be they physical, chemical, biological, or social. But why do we consider this kind of robustness trivial? After all, the robustness of many equilibria we

encountered in genetic systems is very similar in nature. Just take protein conformations or the gene expression patterns of genetic networks: These systems can maintain a functional state despite constant perturbations in the form of thermal noise or gene expression changes.

I surmise that we consider the robust equilibrium (19.2) trivial because it is not part of the system's design by either a human engineer or by natural selection: Neither does such robustness further the purpose of an organism's survival and reproduction in the face of perturbation; nor does it assure that an engineered device continues to function. The chemical equilibrium above just "is." The same holds for countless other systems and their equilibria, from the atomic to the astronomical scale, and even for equilibria in systems whose parts are living things. Examples include the stable population size that logically growing populations eventually attain, the invasion resistance of ecological communities, the stability of governments, and the persistence of human cultures and societies. It is the notion of function and purpose behind robustness—albeit difficult to make precise (326, 470, 631)—that distinguishes robustness in genetic systems from examples like (19.1).

The example (19.1) also leads to a further distinction, a distinction we already encountered earlier. Genetic systems have genetic material on one hand, and other system parts—protein, carbohydrates, etc.—on the other. The ensuing distinction between perturbations to the genetic material and other perturbations—noise and changes in the environment—is specific to genetic systems. In the above chemical reaction, the only possible perturbations are changes of concentrations in the reactants. Such perturbations fall in the category of noise. Changes in the reaction rates  $k_+$  and  $k_-$  would require either a change in the environment or in the reaction mechanism and thus in the reaction (19.1) itself. Thus, whenever we speak of robustness of a system like (19.1) to perturbations, the only possible perturbations are noise and changes in the environment. There is nothing that corresponds to a genetic perturbation.

This second distinction is intimately related to a third distinction that regards the role of natural selection in establishing or increasing robustness in a biological system. Evolution by natural selection has three prerequisites: populations of objects, such as organisms, whose members are able to reproduce; differences in these objects' ability to survive and reproduce; and heritability of these differences, most of which are mediated by genetic material (332). If natural selection influences a biological system's robustness to genetic change, it always does so in populations of organisms with heritable fitness differences. In contrast, most other systems—whether composed of inanimate or animate matter—do not meet these criteria and are not under the influence of natural selection. This holds for chemical systems like (19.1) and for systems as different

from it as ecological communities, human societies, corporations, or governments. Such systems do not form populations and do not reproduce. It is thus not meaningful to speak of heritability and selection among them. If a perturbation disrupts any such system, the system either disappears or is replaced by one with a different organization. Ecological communities—groups of interacting species living in the same area—are no exception, even though they are composed of genetic systems. They do not reproduce like organisms and there is no competition among them leading to a notion of fitness. In addition, the composition of a community can change faster—of the order of years or decades—than its member species evolve.

## Robustness and Self-Organization

The previous paragraph raises an important problem. Natural selection plays no role in changing the robustness of most nongenetic systems, because it plays no role in shaping these systems in general. Nevertheless, such systems, even very simple chemical or physical systems, can attain highly ordered states. They do so through a variety of processes collectively known as self-organization (68, 272, 392). Can self-organization, similar to its role in explaining order in such systems, also help explain their robustness to various perturbations? The answer is yes. I will explain this role of self-organization with a more complex example than (19.1), an example taken from community ecology. I chose this example because a community's parts—individuals from different species—are themselves organisms, yet robustness in communities develops in ways fundamentally different from how robustness evolves in populations of organisms under natural selection.

Community ecology is concerned with the factors that determine which species occur in a community and how abundant its members are. As usual, when studying robustness of a community to perturbations—in ecology often called resilience (71, 242)—it is important to have specific community features and perturbations in mind. Here, I will be concerned with robustness of the coarse community structure—the identity of species in a community. The perturbation at issue is the invasion of a community by new species. This is clearly only one of many perturbations one could study, but it will serve to make the central point.

The literature on robustness of communities to perturbations is vast, and it would be hopeless to even attempt a survey here (212, 362, 443). A characteristic of this literature is that theoretical work is much more advanced than empirical case studies of individual communities. I summarize here a tiny sliver of this work, a class of models of community assembly studied by Richard Law and collaborators (321, 322, 389). Their

key result is that communities tend to develop—not evolve!—increased resistance to invasion of new species over time. This prediction, albeit difficult to test experimentally, is in agreement with other theoretical work in community ecology. Before explaining further the role of self-organization in community assembly, I need to discuss some background material on models of communities and their assembly.

## Modeling Community Assembly

Community assembly is the building of communities through the sequential introduction of species. It is a process that occurs most strikingly in ecological succession, after disturbances have disrupted a community's structure. In extreme cases, such as the aftermath of volcanic eruptions, or the retreat of a glacier, communities have to be assembled from scratch. The species of such assembled communities arrive from a pool of species that occur in the (larger) geographic region that includes the community. If one is interested only in the species that occur in a community, regardless of the abundance of its members, one can represent a community's structure simply as a set of species  $S = \{S_1, S_2, \dots, S_n\}$ . A newly arriving species, say  $S_{n+1}$ , interacts with the species already present. As a result, it may be driven out of the community, drive resident species to extinction, or come to coexist with the resident species. Which of these scenarios will unfold depends largely on how the species interact.

In ecological theory, species interactions are commonly modeled through differential equations of the general form

$$\dot{x}_i = \frac{dx_i}{dt} = x_i f_i(\bar{x}) \quad 1 \leq i \leq n \quad (19.4)$$

where  $x_i$  is the abundance of species  $i$  in the community,  $\dot{x}_i$  is its instantaneous rate of change, and  $f$  is some function of all species abundances  $\bar{x}$  that encapsulates species interactions. The species abundances  $\bar{x}$  can be thought of as a point in an  $n$ -dimensional space, the state space of the community. Over time, the species abundances change, and one or more of the species may become extinct ( $x_i \rightarrow 0$ ).

A prerequisite to study the invasion resistance of communities is a criterion for the stable coexistence of species. One such criterion is the existence of an equilibrium or fixed point of equation 19.4, that is, a species abundance  $\bar{x}$  where all species are present ( $x_i > 0$ ), where their abundances do not change ( $\dot{x}_i = 0$ ), and to which the community returns after sufficiently small changes in abundance. Although widely used, this criterion of stable coexistence has some disadvantages for assessing a community's robustness

to species invasions. First, outside a small neighborhood around a stable equilibrium, the trajectory of the community may approach one of the boundaries of the state space, until one species becomes extinct. This means that such stability—local stability—is of limited use in determining whether species can coexist. In addition, some functions  $f$  may permit no stable equilibrium at all, that is, the species abundances may change periodically or even chaotically, yet all species may coexist indefinitely.

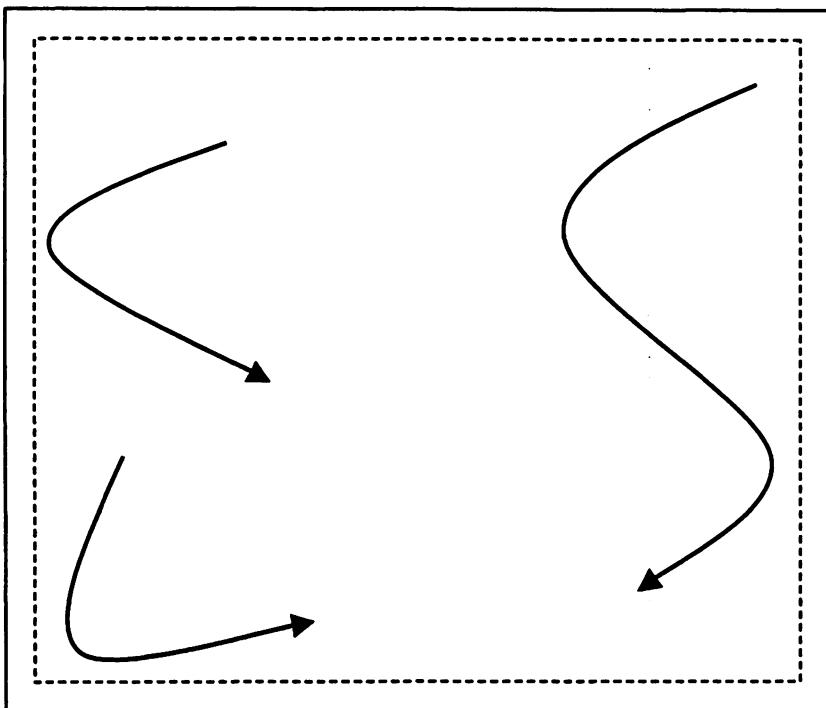
Because of these shortcomings, other criteria of stable coexistence have been defined. One such criterion is that of permanence. Permanence (238) regards the behavior of a community when one of its members is threatened by extinction. Imagine a small zone of some thickness  $\delta > 0$  around the boundary of the space of (19.4). The boundary is the part of the space where at least one of the species abundances is zero. A community is called permanent if it meets the following criteria: First, all abundances  $x_i$  that are outside the zone of thickness  $\delta$  remain outside of it indefinitely (Figure 19.1). Second, any abundance  $x_i$  that is within this zone will eventually increase to a value  $x_i > \delta$ . These two criteria have an important consequence: No one species can become extinct. As one species abundance approaches the zone of thickness  $\delta$ , its abundance will eventually increase. Put differently, the boundary of the space of (19.4) repels the trajectory describing the change of species abundances in time. I here omit mathematically rigorous definitions of permanence and other notions (238, 322), because they are not essential for the main argument.

Whether a model community is permanent depends on the function  $f$  describing the species interactions of (19.4). Unfortunately, even if this function is known precisely, it may be very difficult to prove that a community is permanent. This task becomes easy only if  $f$  is a linear function:

$$f_i(\bar{x}) = r_i + \sum_{j \in S} a_{ij} x_j \quad 1 \leq i \leq n \quad (19.5)$$

Communities (19.4) where  $f$  has this form are said to have Lotka-Volterra dynamics. Here,  $r_i$  is the per capita growth rate of species  $i$  ( $r_i < 0$  if  $i$  is a consumer species and  $r_i > 0$  if  $i$  is a basal species, such as an autotrophic organism), and  $a_{ij}$  defines how strongly species  $j$  influences the growth rate of species  $i$ , for example by consuming species  $j$  ( $a_{ij} < 0$ ). The linear form of (19.5) is not overly restrictive, because many other systems of type (19.4) can be transformed into Lotka-Volterra form (439).

Richard Law and collaborators used the criterion of permanence to study how communities can be assembled through addition of individual species. These investigators used empirical information taken from community



**Figure 19.1** In a permanent community, the abundance of no one species can fall below a minimum value. The solid rectangle stands for the hypothetical state space of a community. The dashed rectangle indicates a boundary layer within this state space, an area where the abundance of at least one species is close to zero, and an area that no trajectory of changing species abundances can enter. Three hypothetical trajectories of species abundances are shown, all of which are repelled by this boundary layer.

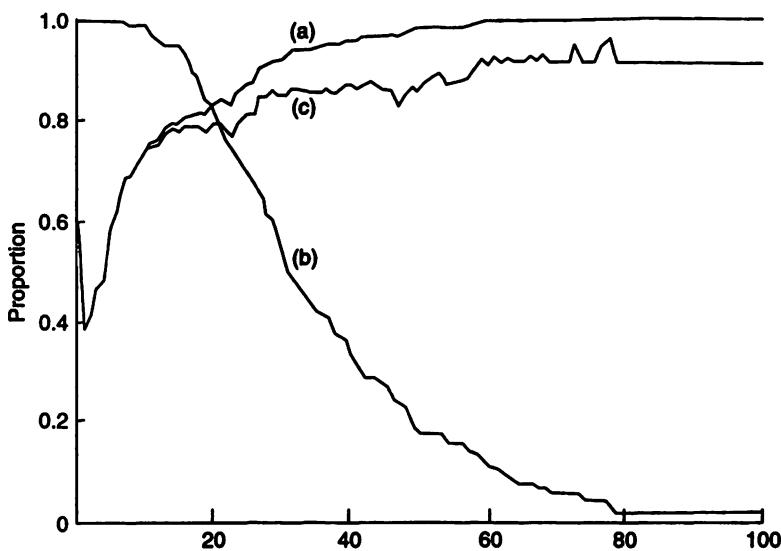
ecology, such as body size distributions and size relations among predators and prey, to make informed choices of growth rates  $r_i$  and interactions  $a_{ij}$  among species. Their approach to studying community assembly begins with a permanent resident community of  $n$  species ( $n \geq 1$ ) and consists of three steps. First, a new species  $S_{n+1}$  is introduced into the community. This species is drawn from a regional species pool whose members are not present in the resident community. The interactions (19.5) of the new species with the resident community can be used to determine whether the new species will be eliminated from the community. If so, the original community remains unchanged. If not, the new species becomes established. In this case, one tests whether the new community that now consists of  $n + 1$

species is permanent. If it is not permanent, one proceeds to a third step, which uses several criteria (389) to determine which smaller, permanent community will be reached through eliminating some of the  $n + 1$  nonpermanent species.

When iterated multiple times, this three-step process leads to the assembly of communities by sequential introduction of new species, a so-called assembly sequence. Each community along this assembly sequence is permanent. Its species can co-exist indefinitely. However, these communities can differ in their resistance to invasion of species that are not in the community. Invasion resistance is the notion of robustness I am concerned with here. A species can invade a community if its abundance  $x_i$  increases when introduced at small abundance into a community. A community's robustness or invasion resistance can be defined as the proportion of (regional) species that are not already in the community and that cannot invade the community. A community is completely invasion resistant if no regional species can invade it. The invasion resistance of communities along a single assembly sequence changes, although it is difficult to predict in which direction. However, when averaged along multiple assembly sequences, a clear trend emerges: On average, invasion resistance increases along assembly sequences (Figure 19.2). That is, communities tend to become more and more invasion resistant.

It is easily understood how species interactions in an established community can prevent the invasion of species. Consider a grassy open landscape with sparse or no tree cover. Such ecosystems contain a great diversity of plant species and support many herbivorous animals. In contrast, woody plants can get established only with great difficulty, simply because the existing herbivores are very effective in removing these plants' seedlings. Put differently, herbivores maintain the grassy open state and prevent invasion of species that might transform the landscape into woodland. Another case in point are coral reefs and their great diversity of species. Herbivorous fish and sea urchins normally keep fast-growing macroalgae in these reefs in check. However, when these consumers are removed through either (human) predation or disease, the macroalgae can proliferate uncontrollably and overwhelm slower growing corals. This is one of the reasons for the degradation of many Caribbean coral reefs (484).

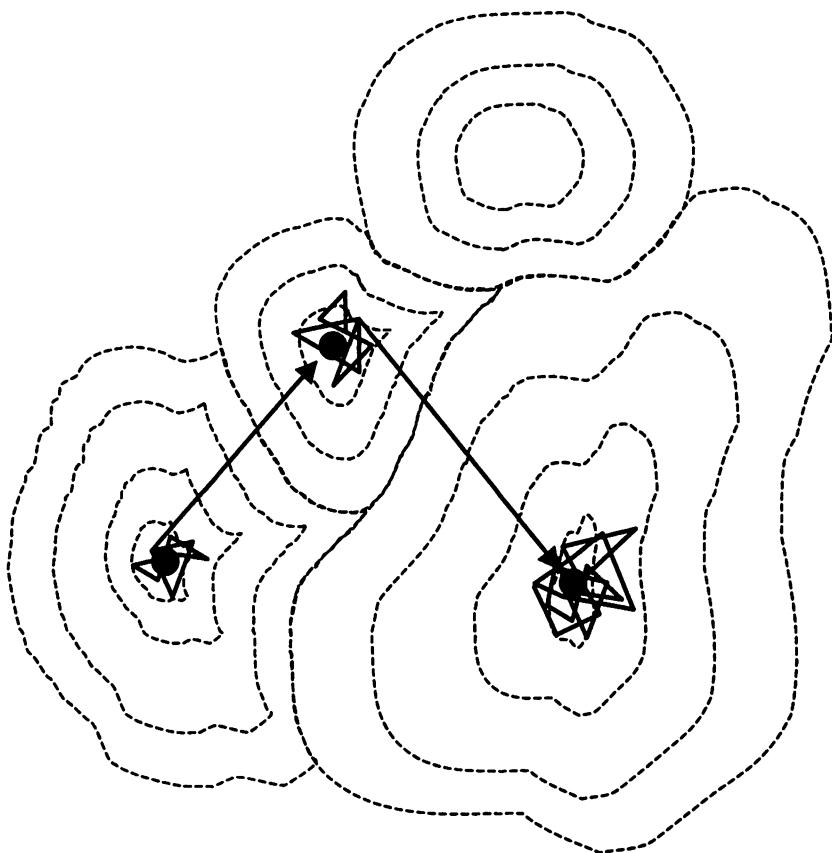
Despite such insights into the mechanisms of invasion resistance, there is no conclusive empirical evidence for the increase in invasion resistance in individual communities. It is easy to understand why such evidence is lacking: Increased invasion resistance occurs as a statistical trend in assembling communities, but there are few systematic studies of community assembly (succession), no replicated studies, and any one community may defy the statistical trend of Figure 19.2.



**Figure 19.2** Average invasion resistance increases during community assembly. The figure is based on 100 assembly sequences from a species pool of five basal and ten consumer species. The horizontal axis shows the number of species introductions in any one assembly sequence. The vertical axis shows the proportion of 100 assembly sequences with a given property. (a) The average invasion resistance among the assembly sequences, that is, the average proportion of species in the region that cannot invade. (b) The proportion of assembly sequences that have yet to reach a completely invasion resistant state. (c) The average invasion resistance among only the assembly sequences that are not completely invasion resistant. From Figure 5.6. in (321).

### Increased Robustness Through Self-Organization

How can we understand the increase of invasion resistance documented in Figure 19.2? Natural selection clearly has nothing to do with it, because the conditions under which selection operates do not exist. Clearly, it is random change itself—the invasion of nonresident species—that drives these communities to a state of higher invasion resistance. To visualize this process, it is tempting to resort to the well-worn visual metaphor shown in Figure 19.3. The figure partitions a “space” of possible community compositions into multiple regions (valleys) separated by ridges. Each region corresponds to a permanent community, and the larger the valley associated with a region, the more invasion resistant the corresponding community is. During an assembly sequence, a community (indicated by a dot in the figure) starts out in one of the regions. The formation of a new permanent



**Figure 19.3** A visual metaphor to illustrate how a system can increase robustness to perturbations through those very perturbations. The figure shows a contour map of a space of possible system states that is subdivided into four valleys of different sizes. The larger a valley, the more perturbation-resistant the system is when in the corresponding state. The system's state is represented by a black ball that is driven from valley to valley through a series of perturbations. It eventually arrives at the largest valley, the most robust state. The time spent in each state will be proportional to the robustness of the state, such that the system will spend most time in the most robust state(s). Note that in this scenario, a system can develop robustness only to perturbations it has actually encountered in its history.

community through introduction of a new species corresponds to a transition between these regions. Which of the regions is accessed by each transition depends on the introduced species and is thus unforeseeable.

This metaphor is appealing for several reasons. First, it illustrates how communities starting in a state of low invasion resistance can attain high

invasion resistance. The reason is that once a community resides in a region with high invasion resistance, it will simply spend more time in that region, precisely because of its high invasion resistance. Second, the metaphor shows that increased invasion resistance cannot be a feature of just one assembly sequence, because any one community can transition from a state of high to low invasion resistance. Only on average will different communities accumulate in regions of high invasion resistance. Third, the metaphor is reminiscent of landscapes that occur in numerous other processes, such as the landscape of free energies in folding proteins. Valleys in this landscape correspond to stable conformations, and proteins transition between valleys through thermal noise. The widest and deepest valley corresponds to the native and most robust conformation. From this perspective, a community's approach of an invasion resistant state is analogous to a protein's approach of its native and most stable conformation.

The metaphor of Figure 19.3, although it captures essential features of the process, also has serious limitations. Most importantly, although changing species abundances *within* a community are easily represented in some (high-dimensional) space defined by (19.4), matters are no longer that straightforward for communities whose species composition changes. For instance, the introduction of a new species into an  $n$ -species community increases the dimension of the relevant space from  $n$  to  $n + 1$ . Thus, Figure 19.3 is misleading in that the state space dimension changes during community assembly. The second problem is that communities can eventually attain complete invasion resistance. No species in the region can invade them. Figure 19.3 captures this scenario poorly, because it insinuates that communities can escape from any region of the space, even from the one with highest invasion resistance. I thus briefly discuss a better—albeit less pictorial—representation of the space in which community assembly occurs. Importantly, this representation leaves the key concept intact: noise drives communities toward increased robustness.

### A Graph-Based Metaphor for Self-Organized Community Robustness

If there are  $N$  species in a regional species pool, then we can represent every community—permanent or not—that these species can form by a binary vector  $\vec{S}$  of length  $N$ , where entries of 1 and 0 at position  $k$  ( $1 \leq k \leq N$ ) indicate the presence or absence of species  $k$  in the community. Taken together, all such vectors constitute a discrete space of possible community compositions. The distance of two communities in this space corresponds to the number of species in which they differ. Consider

now only the points in this space that correspond to permanent communities, and consider a graph whose nodes are these points. Connect any two nodes  $\bar{S}$  and  $\bar{T}$  in this graph by a directed edge ( $\bar{S} \rightarrow \bar{T}$ ) if the permanent community  $\bar{T}$  can be reached from  $\bar{S}$  by introduction of a single species. (Note that  $\bar{T}$  is not necessarily the same as  $\bar{S}$  to which the introduced species has been added, because the introduced species may lead to the extinction of other species.) The structure of this graph will depend on the details of the interactions between species. The analogy to the sequence space of macromolecules and the graphs connecting molecules with stable conformations (or the same conformation) are immediately obvious. However, a difference with important consequences is that the graph I just described is directed: Even though  $\bar{T}$  may be reachable from  $\bar{S}$ ,  $\bar{S}$  may be unreachable from  $\bar{T}$ . This implies that completely invasion-resistant communities may exist. No edges emanate from such communities, and these communities are maximally robust endpoints of any assembly path. In contrast, other graphs I discussed, such as the graph of protein sequences adopting the same conformation, have no such end points. The reason is that any (mutational) change, converting one point in sequence space to another, can be reversed. In other words, it is the directionality of the community assembly graph that makes complete invasion resistance—perfect robustness—possible.

In principle, changes in community structure and invasion resistance in such community graphs can be easily predicted with standard mathematical methods such as Markov chains (270). Such a calculation could answer numerous questions about how species introductions change community robustness. For example, are nearby and thus similar communities more likely to be connected than distant communities? This would mean that the average species introduction does not cause radical changes in community structure. Are “absorbing” nodes that are completely invasion resistant rare or frequent? (In the model communities studied by Law and collaborators most assembly sequences eventually terminate at such absorbing nodes (321, 389).) In practice, however, such a graph-based approach to understanding community assembly would be difficult, because the graph’s structure depends on a myriad of unknown details on species interactions, and on the unknown frequency of species invasions.

Despite these substantial knowledge gaps, the example of community assembly serves to illustrate one central point. Perturbations in various forms can push a system toward increased robustness to the very same perturbations it encounters. The reason is that the system will spend the most time in the state(s) that are most resistant to such perturbations. The same principle applies to many systems above and beyond the organism. It also applies, with an important modification, to systems inside

organisms. An illustrative example closest in spirit to community assembly is the self-assembly of macromolecular complexes. Every cell contains thousands such complexes. Some of these complexes contain few macromolecules, others, like the spliceosome or the transcriptional machinery, dozens to hundreds. In their assembly, some complexes may change continually through dissociation and re-association of their components, until a final, most stable and functional composition has been reached. Similarly, in protein folding, thermal noise drives amino acid chains to explore various conformations until the native conformation that is most robust to such noise has been reached. In a key difference to nongenetic systems, however, natural selection may have shaped these systems' composition—the landscape of Figure 19.3—such as to ensure rapid and effective complex assembly or folding without stable yet nonfunctional intermediates.

In sum, robustness to perturbations in inanimate systems—from physics to economics—differs in two important ways from the robustness of genetic systems to genetic change. First, robustness to genetic change does not exist in inanimate systems, because such systems have no genetic material. Second, inanimate systems do not fulfill key criteria that would permit natural selection to change their robustness. However, robustness in such systems can increase through self-organization driven by random perturbations to the system. The result of this process can be encapsulated in one simple phrase. A system will spend most of its time in a robust state, precisely because such a state is more robust. Put differently, if the world is rife with robust systems, it is because fragile systems are fleeting.

## Robustness in Man-made Systems

Biological systems perform complex tasks. But so do nonliving engineered systems. Are the principles that govern their design different? Or are they fundamentally similar? Do they achieve robustness in similar or different ways? The answers to these questions could fill another book. In this chapter—really more of an afterthought—I make a few comparisons between living and nonliving engineered systems. I first point out two fundamental differences in their architecture, differences that have implications for questions related to robustness. These are the erratic behavior of parts of many biological systems, as well as the apparently unnecessarily complex architecture of such systems. However, I also argue that these apparent differences may obscure even more important similarities. I discuss a few such similarities, focusing in particular on one source of robustness that harks back to a topic I discussed earlier, distributed robustness. In a final example, I show that even a feature that one might think is unique to biological systems, evolved robustness, can be implemented in engineered systems.

To begin with, a distinction: Engineered systems contain nothing comparable to genetic material. Strictly speaking, it is therefore also impossible to compare, say, the failure of a transistor on an integrated circuit with a mutation in a gene, or with a change in a protein’s phosphorylation state. Nevertheless, a distinction related to that between genetic and other perturbations is as important for engineered devices as it is for the living. It is the distinction between perturbations to a system’s parts and changes in a system’s surroundings. Perturbations in a system’s surroundings may encompass changes not foreseen in its design, such as extreme temperatures, but also changes that are at the very heart of its function, such as the temperature changes that a thermostat is designed to control. In contrast to such external changes, alterations in a system’s parts do change the architecture of the system itself. In this sense, failure of parts is the closest counterpart of genetic change in engineered systems. In the engineering literature, devices that continue to perform their function in the face of part failures are usually called fault-tolerant. To remain consistent with previous chapters, however, I will continue to use the word robustness for such fault-tolerance. It would be impossible to

offer a brief survey of the massive engineering literature—hundreds of books and several journals (1, 2, 4)—devoted to this topic. Therefore, I focus here on a few illustrative examples.

### Some Apparent Design Differences and Their Consequences for Robustness

Here are two of the most glaring differences between living and nonliving engineered systems. The substrates of many engineered systems—steel, plastics, silicon—are fundamentally different from the proteins and nucleic acids of living systems; and biological systems have come about not through rational design, but through the blind groping search of biological evolution. But are there differences beyond such commonplaces? Yes. And among these differences (307), the following two are most obviously related to the theme of robustness.

First, the parts of living systems behave much more erratically than the parts of most engineered systems. Individual proteins wiggle randomly between multiple conformations; the number of protein molecules produced from any one gene fluctuates randomly; cells take different amounts of time to divide; and neurons fire at erratic intervals. Despite such noisy behavior, many biological processes have predictable outcomes. One would thus think that organisms may have evolved special mechanisms, mechanisms not found in engineered systems, to suppress such noise or to channel it into harmless forms. (Noise suppression would have mutational robustness as a by-product, as I argued earlier.) However, no radically new approaches are needed to deal with noise inside organisms. To see this, consider first that both engineered and biological systems are exposed to frequent changes in the external world. And although internal noise may be less important in engineered systems, both types of change can affect a system very similarly. Thus, similar mechanisms can deal with either kind of change. Just consider the well-worn example of the feedback loop. It can allow a system to adjust its behavior to changes in the external world, such as changes in temperature or osmotic pressure. But equally well, it can reduce the sensitivity of a system to internal noise, such as random fluctuations in regulator molecules (175). Thus, although organisms are unique in the erratic behavior of their parts, they may not need unique design features to reduce adverse effects of this behavior.

A second feature is that many organismal systems perform their functions in complicated, apparently inelegant and outright byzantine ways. The paradigmatic example is gene regulatory networks in embryonic development, such as the network that patterns the fruit fly embryo (7). This network is responsible for subdividing the embryo into different segments.

The task is accomplished through the expression of key developmental genes in a striped pattern reflecting the prospective segments. Physicists have long recognized that there are very simple ways to achieve such striped gene expression patterns, ways that involve no more than two different regulatory molecules (188). Instead, flies employ more than a dozen genes that regulate each others activity in very complicated ways to drive the striped gene expression necessary for segmentation. Why this complexity? An engineer might have designed an embryonic patterning device very differently and certainly more elegantly.

There are two possible reasons. First, much like a medieval city, which is an erratic jumble of houses and streets, such inelegance may result from an organism's billion-year-long history. In other words, it may be a product of history with no further biological significance. But there is another possibility: This inelegance may be necessary to render a biological system robust (70, 307, 607). For instance, the byzantine architecture of a segmentation gene network may be necessary to ensure consistent embryogenesis in the face of variation in temperature, embryo size, concentrations of regulatory molecules, and perhaps even mutations. But, again, if this hypothesis is true, then engineered systems are not exempt from it. For example, while it may be simple to design a minimal jet aircraft, it is a different matter to build a commercial airliner whose performance is robust to variation in payload, weather conditions, and faults in its components. It is thus perhaps no coincidence that modern commercial aircraft consist of more than 100,000 different subsystems and employ more than 1000 central processing units that automate the aircraft's operations (70). In other words, while it may be possible to design engineered systems simply and elegantly, robust engineered systems may be no simpler than biological systems. These two examples—noisy parts and complex architecture—indicate that some differences between the architecture of living and nonliving systems that appear fundamental at first, do not require fundamentally different ways of thinking about robustness. The following examples further underscore this point, while at the same time speaking to central themes of this book.

### Distributed Robustness and Telecommunication Networks

As we have seen earlier, there are two principal means to render organisms robust to genetic perturbations: Redundancy of parts (genes), and the distributed robustness exemplified by most of the examples in this book. Among the two, redundancy of parts is easiest to understand and implement. The principle is as simple as keeping spare light bulbs in the house. Nevertheless, it is also used in the most complex endeavors, such

as in space missions that are guided by redundant computer systems. The engineering literature heavily emphasizes the use of redundancy to ensure robustness (113, 273). Because redundancy of parts is easy to understand in principle, I will spend no further time on it here. Instead I will focus on the second kind of robustness, distributed robustness. In biological systems, distributed robustness is at least as important as redundancy. However, the straightforward application and great success of parts redundancy in engineering invites the question whether distributed robustness has a place in engineered systems at all. My first example, taken from one of the largest technological system created by humans, shows that it does. The second example—involving some of the smallest systems, integrated circuits—also serves to illustrate distributed robustness, but goes one step further. It demonstrates the use of evolutionary principles in designing engineered systems. Specifically, the example will show that selection can render an engineered system robust to failures of its parts.

The global public switched telephone network, together with the internet, is the largest technological system ever created. The telephone network's primary purpose is very simple: to transmit voice information between any two points. At the heart of this network is a complex device that gives the network its name: the telephone switch. Telephone switches enable telephone sets to communicate with each other. The three basic functions of a telephone switch are simple in principle: generate a dial tone when a calling party lifts a receiver, route (switch) a call to the called telephone set, and disconnect the route once one of the two parties terminates the call. However, to perform these functions reliably for hundreds of millions of customers requires sophisticated technology. Even the most basic functions of a telephone switch involve hundreds of thousands of lines of computer code. In addition, in the United States alone, thousands of individual switches are linked in a sophisticated network with complex topology.

Despite such complexity, the U.S. public switched telephone network is highly reliable. A systematic study of factors causing network failures in a two-year period between 1992 and 1994 shows that the network is available more than 99.999% of the time (314). Network failures are conveniently measured in customer minutes—the number of customers affected by an outage multiplied by the number of minutes the outage lasts. Hardware failures are responsible for only 7% of lost customer minutes, and software failures only for 2% (314). The vast majority of failures stem from human error and network overloads. (Overloads are outages anticipated in the network's design. Telephone companies could reduce their frequency, but only at prohibitive costs.)

What is responsible for such extreme reliability and for the low outage rates due to failures in network components? The high reliability of

individual telephone switches is part of the answer. For instance AT&T telephone switches are expected to experience no more than 2 hours of failure in 40 years, a failure probability of  $5.7 \times 10^{-6}$  per hour (645). (I note parenthetically that this failure rate is not much greater than genic mutation rates in some organisms (123).) The factors responsible for such robustness include redundant hardware and recovery software. However, another key element of robustness is the distributed nature of the network itself. Because failures of network components are local, telephone switches can reroute traffic dynamically to avoid a failed switch. The local nature of failures also implies that such failures usually have little impact on the network as a whole.

This is a prime example of distributed robustness: Although telephone switches do similar things, the network topology in which they are embedded and their different positions in this topology give them different roles. Specifically, they route different calls. The very fact that they are embedded in a large network also allows the dynamic rerouting of calls and hence robustness to switch failure. A price paid for the ability to reroute traffic dynamically is that telephone switching centers need to store global information about alternative paths throughout the network and exchange information on the status of individual switches continually. Such global interactions themselves can lead to some system failures (314).

The second behemoth among telecommunication networks, the internet, has a radically different architecture. The internet is “packet-switched,” breaking up a stream of data into individual packets transmitted over a path shared by many users, whereas the telephone network is “circuit-switched”—every call uses a dedicated circuit that cannot be used by anybody else. Also, most of the telephone network’s “intelligence” is contained in its switches, whereas most of the internet’s intelligence is contained in its end points, individual computers. Nonetheless, the internet’s robustness to failed network components—almost as high as for the telephone network—relies heavily on the same ability to dynamically reroute data packets. Such distributed robustness was already a central design requirement for the internet’s predecessors (613).

This example of distributed robustness in telecommunication networks illustrates that robustness through redundancy of parts is not the only means by which robustness is achieved in man-made systems. However, both telephone networks and the internet are very large systems. In addition, their very size and growth over many years implies that no one individual or institutional decision determined their current structure. Is distributed robustness restricted to such systems, systems that are very large, and systems whose design lacks the element of complete premeditation? The following example, while focusing on the evolution of robustness, shows that system size does not matter. Electronic circuits, among

the smallest engineered systems, can also show distributed robustness. In addition, the example also suggests that the absence of completely rational, premeditated system design may favor the origin of distributed robustness.

## Evolved Integrated Circuits

Evolutionary principles can be used to develop devices that perform a specific computation (279). The same principles can also render a device robust to failure in its parts. Such “evolved” devices are examples of evolvable hardware (477, 501), hardware that can be designed and modified using principles borrowed from biological evolution. The electronic circuit in Figure 20.1 is an example of evolvable hardware. It is a field-programmable transistor array. A transistor array consists of multiple transistors arranged on a two-dimensional substrate. The transistors are connected through a series of switches, each of which can be either closed or open. These switches are “field-programmable,” that is, they can be easily closed or opened by a user. The current flowing through the transistor array depends on the status of these switches. Put differently, the configuration of these switches defines an electronic circuit that carries out a computation when exposed to some input current. (These switches are often themselves implemented by transistors that are not considered part of the circuit.)

Their easy modification makes field-programmable transistor arrays ideal to explore alternative circuit designs, compare their performance in solving a particular computational task, and identify the best circuit among them. Various approaches exist for such circuit optimization. One of these approaches, genetic algorithms, explicitly use evolutionary principles gleaned from biology to find optimum solutions to engineering problems (240). Genetic algorithms represent various solutions to a problem in a population of strings. Each string—also known as a “chromosome”—encodes one potential solution to the problem. All strings in a population undergo “mutation”—changes of individual letters—and “recombination”—swapping of contiguous segments among strings. In addition, the strings are subject to selection. That is, strings encoding superior solutions to the problem survive preferentially into a next “generation.” Over multiple generations of mutation, recombination, and selection, a genetic algorithm can find one string that best solves the problem at hand.

In a genetic algorithm, one best represents an electronic circuit implemented by a field-programmable transistor array through the status of the array’s switches. That is, each possible circuit is represented by a

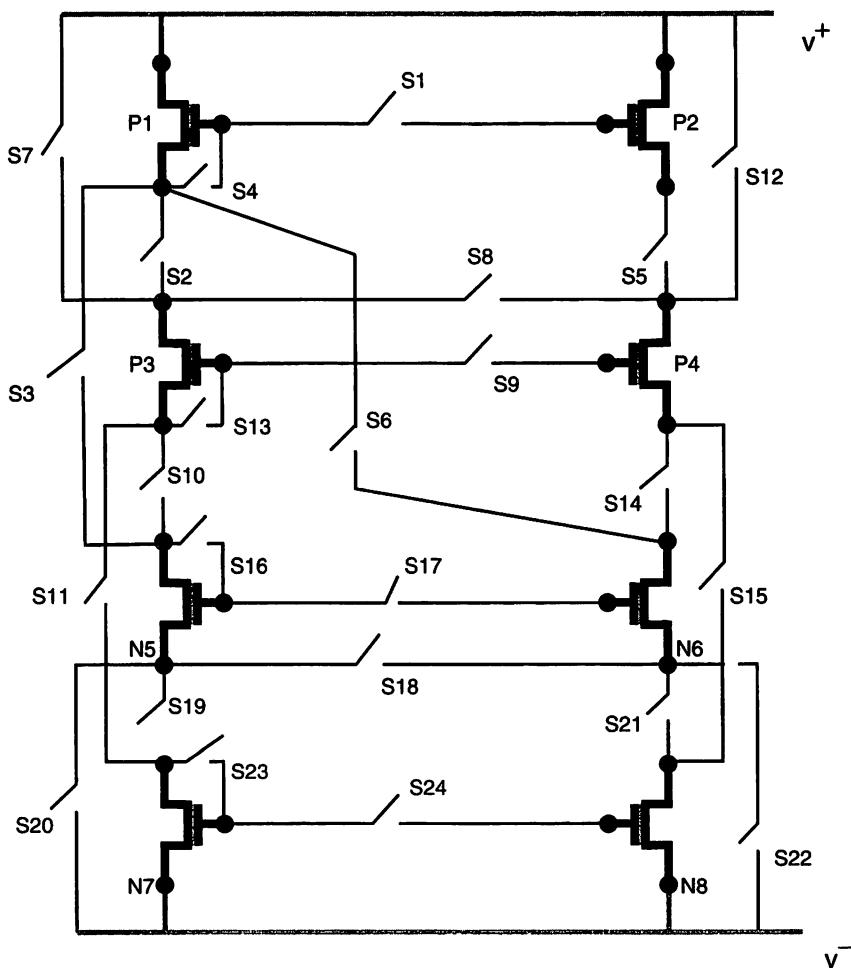


Figure 20.1 A field-programmable transistor array. Eight transistors (labeled P1–P4 and N5–N8) are connected by 24 field programmable switches  $S_i$ . From Figure 1 in (279). © 2000 IEEE.

string of ones and zeroes. Each position on the string corresponds to the status of just one switch—open (0) or closed (1). Notice that even the simple circuit of eight transistors shown in Figure 20.1 has more than  $10^7$  possible switch configurations.

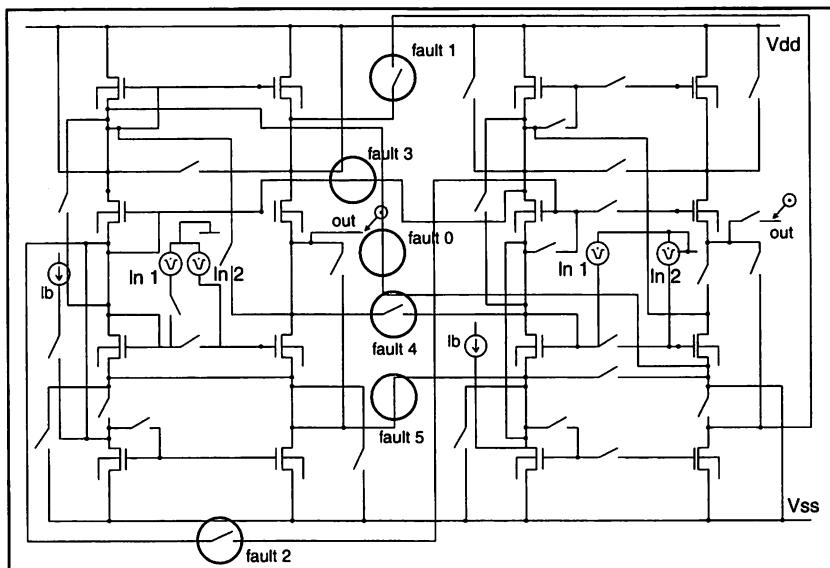
In two separate experiments, Keymeulen and collaborators (279) used a genetic algorithm to find electronic circuits that perform two simple computational tasks. Each experiment employed two identical field-programmable transistor arrays, each of which consisted of 8 transistors

and 24 programmable switches (Figure 20.1). The two circuits were interconnected through 6 external wires, which were themselves equipped with programmable switches. Two input voltages,  $In_1$  and  $In_2$ , were applied to these circuits in prespecified locations. One output voltage,  $Out$ , constituted the result of the computation. The experiment aimed at circuits that could perform the following two very different computations. The first is an analog multiplication, where the circuits output voltage is the product of the input voltages, i.e.,  $Out = In_1 \times In_2$ . The second computation is the logical XNOR function, which assumes a value of one if both input voltages are high and if both input voltages are low:  $Out = \text{XNOR}(In_1, In_2)$ . A given switch configuration's performance was measured by the mean-squared distance between the actual output and the desired output of the circuit. For example, for the XNOR function, the performance of a circuit was determined by the average of  $[\text{XNOR}(In_1, In_2) - Out]^2$  over four possible states of the input voltages,  $(0, 0)$ ,  $(0, 1)$ ,  $(1, 0)$ , and  $(1, 1)$ , where a value of one indicates a high input voltage. The smaller this average mean-squared distance, the better the circuit performed. Performance was measured either in an actual circuit implementation on a field-programmable transistor array or through a numerical simulation of circuit behavior.

## Robustness in Evolved Integrated Circuits

A genetic algorithm operating on a population of 200 strings found a circuit that performed the XNOR function after 60 generations of simulated evolution. Figure 20.2 shows a sketch of this circuit. Keymeulen and collaborators determined how robust this circuit was to faulty switches (279). Specifically, they evaluated the performance of the circuit to changing the state of the six switches (circled in Figure 20.2) that connect the two field-programmable transistor arrays. Three of these switches are normally open, implying that they would be closed in a faulty state. Conversely, the other three switches are normally closed, but they remained open in the faulty state. The best circuit calculated the XNOR function correctly for two of these faults, but failed to do so for the remaining four. In this sense, the circuit was robust to failure of two switches, failures that it had never encountered in its evolutionary history. However, it was not robust to failure in four other switches.

The population of strings in generation 60, where the best-performing circuit first occurred, also contained some circuit variants that performed as well as the best circuit without any faulty switches. Importantly, some of these variants—when containing faulty switches—performed better than the best circuit when faced with the same switch faults. Such



**Figure 20.2** An electronic circuit, evolved through a genetic algorithm to compute the XNOR function. Each half (left and right) of this circuit corresponds to one field-programmable transistor array shown in Figure 20.1. The circled switches correspond to the connections between two arrays. They are also the switches for which fault-tolerance is assessed. From Figure 11 in (279). Copyright 2000 IEEE.

variation in robustness suggests that robustness itself can evolve in electronic circuits, without impairing the circuit's performance under faultless conditions. A variation of the above evolution experiment confirms this conjecture. Specifically, Keymeulen and collaborators repeated the circuit evolution experiment under conditions where performance of each circuit is evaluated not only in the intact state, but under the faulty switch configurations listed above. After 60 generations of simulated evolution under these more stringent conditions, the circuit correctly computes the XNOR function both with all switches intact, and under conditions where any one of six switches failed. Thus, when forced to operate with faulty switches during its evolution, a circuit can evolve robustness against these faults. In relation to the previous examples, I mention parenthetically that such robustness does not simple stem from a redundancy of parts. Although the transistors in a transistor array are “redundant” in the sense that there are many of them and they are identical, fault tolerance emerges from a global configuration of switches and not from any duplication of small circuit elements that have identical functions.

Evolved robustness to faulty parts is not a peculiarity of circuits calculating the XNOR function. The same is observed for other circuits, such as circuits performing analog multiplication. Without encountering faulty parts during its evolution, an evolved analog multiplier circuit is robust to some faulty parts, but not to others (279). However, when evolving under conditions where these faults occur, the circuit evolves robustness to them. Variation in robustness to parts failure and evolution of robustness has also been demonstrated in an inverter circuit (a NOT gate), an amplifier, an oscillator, and an electronic controller for a mobile robot (548–550).

In general, evolved circuits often differ in one key respect from rationally designed circuits. In rationally designed circuits, it is usually easy to decompose the circuit into parts that carry out specific functions. In contrast, evolved circuits often do not show such a decomposition (548, 549).

The example of circuit evolution also demonstrates the advantages of the controlled conditions under which one can evolve artificial systems like electronic circuits. One of these advantages is that one can introduce specific defects at arbitrarily high rates during the evolution process. In contrast, mutations in living systems occur at random positions and usually at very low rates. As I discussed earlier, low mutation rates can prevent mutational robustness from evolving unless population sizes are very large. The human engineer also has the luxury of biasing the mutation process towards specific premeditated faults, thus repeatedly evaluating a system's performance under these faults. This can render the device maximally robust against the faults.

The above examples demonstrate what may be obvious in hindsight—that the same evolutionary principles apply to evolving organisms and to electronic circuits. Both distributed robustness and its evolution can play as important a role in engineered systems as in biological systems. The examples also show how insight into the evolution of robustness in biological systems can assist in evolving devices that are robust to a defined spectrum of faults. The simplest such insight is that of a minimum necessary rate of either random or targeted “mutations.” Beyond these few and simple answers, however, many questions that remain open in engineered systems are similar to those we find in biological systems. What are the limits of evolving robustness in engineered systems? How do these limits depend on the kind of system, its size, and the number of anticipated faults? What trade-offs exist between robustness and other aspects of system performance?

It may be easier to answer these questions for engineered systems than for systems inside living organisms. First, the behavior of engineered systems is more easily measured and controlled—a property that is part of

their design. And, second, the evolutionary process shaping them can be fine-tuned in the laboratory. Thus, in the long run, not only may engineers learn from biology, but biologists may take advantage of the controlled environments offered by engineers to learn more about the origins of biological robustness.

# *Epilogue*

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## Seven Open Questions for Systems Biology

The following are key open questions and problems that emerged from the preceding chapters. We will understand mutational robustness to the extent that we answer these questions for a wide variety of biological systems. Currently, however, none of these questions have conclusive answers in any system. I note that most of these questions are empirical, not theoretical questions. This reflects the view that major theoretical developments in this area are in place, but that empirical data are still sorely lacking.

- *Which of the two main evolutionary causes of robustness is most important?* The first cause comes into play whenever evolution blindly searches for optimal solutions to problems that organisms need to solve to survive and reproduce (chapter 13). In such a search, solutions (systems) with a large associated neutral space will be preferentially discovered, because such solutions are frequent among all possible solutions. At the same time, such solutions correspond to robust biological systems. The second principal cause is incremental evolution of robustness within a neutral space, a process that can find regions within a neutral space where robustness is especially high (chapters 13, 16, 17). We do not know whether the robustness we observe is primarily a by-product of the evolutionary discovery process, or whether it results from incremental evolution within a neutral space.
- *Is it possible to infer the global structure of a neutral space from a small sample, a small number of biological systems within the space?* Such methods are important because most neutral spaces are vast and we cannot hope to characterize them exhaustively, but understanding the structure of neutral spaces is critical to understanding the robustness of biological systems and to what extent this robustness can itself evolve.
- *What concrete empirical examples exist of mutations that were once neutral but later gave rise to important evolutionary innovations?* As I argued earlier (chapter 14), a mutation’s effect on a biological system depends very much on the context—environmental or genetic—in which the mutation occurs. Almost all mutations that are neutral in one context can affect a system’s behavior in another context. Although it is clear that this feature of

robust systems can promote evolutionary innovation (chapter 14), we have only tentative examples for neutral mutations that gave rise to profound evolutionary innovations.

- *Is robustness to mutations ever an adaptation to mutations?* We saw (chapters 15 and 16) that mutational robustness is associated with robustness to nongenetic change, that is, environmental change and noise. I also argued that selection for robustness to mutations is weaker than selection for robustness to nongenetic change. This raises the possibility that wherever natural selection increased the mutational robustness of a system, it did so in response to the harmful effects of nongenetic change. Are there *any* systems where mutations were the primary driving force for the evolution of mutational robustness?
- *Is robustness always associated with other features of biological systems that facilitate evolvability?* Robustness is not the only feature of biological systems that facilitates evolvability (287). I briefly discussed one other such feature, modularity, the decomposability of biological systems into parts with distinguishable structure and semiautonomous functions. Modularity clearly promotes evolvability, but, as I briefly discussed in chapter 6, arguments exist both in favor and against the possibility that modular systems are necessarily also robust (chapter 6).
- *How is robustness constrained by other aspects of a biological system's performance?* In chapter 18, I proposed a mathematical framework to analyze the trade-offs that mutational robustness faces. I also presented some empirical examples of known trade-offs, such as that between mutational robustness and reproduction speed in viruses, or biochemical activity in enzymes. All these examples are, unfortunately, qualitative: We do know that such trade-offs exist, but they have not been characterized quantitatively in any system.
- *Where has natural selection promoted fragility over robustness?* Some biological systems I discussed in chapter 18 are not robust but fragile. That is, they generate much phenotypic variation in response to mutations. (Such an increase in fragility is different from an increase of mutation rates as a mechanism to increase variation.) Fragility may have evolved because it is sometimes advantageous. Fragility, however, cannot evolve through individual-based selection, because it confers no advantage on the individual cell or organism, but only on groups, lineages, or populations of cells and organisms. Its evolution through natural selection can thus occur only in a limited number of circumstances. Chapter 17 listed some candidate examples for such evolved fragility, but in neither of them has natural selection for increased fragility been demonstrated conclusively.

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# Robustness and Evolvability in Living Systems

Andreas Wagner

All living things are amazingly complex, yet their DNA is unstable, undergoing countless random mutations over many generations. Despite this instability, most animals do not die, plants continue to thrive, and bacteria continue to divide. Not only has life persisted in the face of change, it has produced an endless stream of new forms. *Robustness and Evolvability in Living Systems* tackles this perplexing paradox. The book explores why genetic changes do not cause organisms to fail catastrophically and how evolution shapes organisms' robustness. Andreas Wagner looks at this problem from the ground up, starting with the alphabet of DNA, the genetic code, RNA, and protein molecules, moving on to genetic networks and embryonic development, and working up to whole organisms. He then develops an evolutionary explanation for robustness.

Unparalleled in its field, this book offers the most detailed analysis available of all facets of robustness within organisms. It will appeal not only to biologists but also to engineers interested in the design of robust systems and to social scientists concerned with robustness in human communities and populations.

"Wagner contributes significantly to the emerging view that natural selection is just one, and maybe not even the most fundamental, source of biological order."

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