Robustness of Cellular Functions

Review

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Summary

Robustness, the ability to maintain performance in the face of perturbations and uncertainty, is a long-recognized key property of living systems. Owing to intimate links to cellular complexity, however, its molecular and cellular basis has only recently begun to be understood. Theoretical approaches to complex engineered systems can provide guidelines for investigating cellular robustness because biology and engineering employ a common set of basic mechanisms in different combinations. Robustness may be a key to understanding cellular complexity, elucidating design principles, and fostering closer interactions between experimentation and theory.

Introduction

Biological systems maintain phenotypic stability in the face of diverse perturbations arising from environmental changes, stochastic events (or intracellular noise), and genetic variation. It has long been recognized that this robustness is an inherent property of all biological systems and is strongly favored by evolution (de Visser et al., 2003). In development, robustness is described as "canalization" toward a specific outcome from uncertain starting conditions (Waddington, 1942). Despite this central role in biology, there is still a limited understanding of what robustness precisely is and how it is accomplished at the cellular or molecular level (Hartman et al., 2001). A major reason is that robustness and the apparent complexity of cellular systems are intimately linked and, therefore, both are difficult to understand (Lauffenburger, 2000; Carlson and Doyle, 2002). During the recent "rediscovery" of robustness, however, formal approaches, such as mathematical modeling, gained

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new momentum, and these will play a central role in addressing this complexity. Apparently, mathematical models in biology as well as "network cartoons" are idealizations and not perfect representations of reality. Their value is to be judged by the novel insights they bring to our understanding of cellular complexity.

Formal approaches require and entail a precise representation and unbiased manipulation of knowledge. Once biological knowledge is formalized, the rules of mathematics take over; this may often provide a fresh insight into biology (Endy and Brent, 2001; May, 2004). For instance, the suggestion that adaptation to constant stimuli by the bacterial chemotaxis system is a robust property of the network, and not a result of fine-tuning of biochemical parameters, originated from studies of a simplified mathematical model (Barkai and Leibler, 1997). Detailed theoretical and experimental analysis later confirmed this proposition (Alon et al., 1999). Likewise, signaling pathways (Huang and Ferrell, 1996; Lee et al., 2003), cell cycle regulation (Borisuk and Tyson, 1998; Morohashi et al., 2002; Pomerening et al., 2003), and developmental control circuits (von Dassow et al., 2000; Meir et al., 2002; Eldar et al., 2002, 2003) displayed aspects of robust behavior in silico as well as in vivo. In many of these examples, moreover, model development led to new biological understanding validated by experiment.

As these examples illustrate, understanding the design principles ensuring robustness requires an analysis of degrees of complexity that often cannot be grasped by intuition alone. This bears similarities to the situation for highly evolved engineered systems with complex control circuits guaranteeing reliable performance of vital functions under conditions of uncertainty (Csete and Doyle, 2002). In this review, we therefore focus on general design principles that underlie robustness in biological systems and their role for gaining a deeper understanding of cellular complexity. We will discuss the relevance of understanding robustness—What is the tangible outcome of studying this issue for life sciences? Such an overarching concept as robustness will certainly play several roles in biological research. It can be viewed as an overall evolutionary design principle or a scientific approach. More optimistically, it may be the panacea to the ailments affecting large-scale dynamic modeling of biological systems. At the least, in the hands of pragmatic researchers it can function as a tool producing testable biological hypotheses.

The Concept of Robustness

In general, robustness means the persistence of a system's characteristic behavior under perturbations or conditions of uncertainty. Already at the level of exact definitions, robustness is a complicated topic. We argue that assessment of robustness requires that one be precise about the behavior that is robust and on the nature of uncertainty; robustness properties can drastically change by altered specifications (Stelling et al., 2004). Even in theoretical engineering disciplines, however,

various mathematical characterizations exist that are not exactly equivalent (Cover and Thomas, 1991; Zhou et al., 1995), which highlights the need for conceptual advances. In biology, the concept of robustness closely relates to "stability," "homeostasis," and "canalization," but it covers a broader class of phenomena (Kitano, 2002; Carlson and Doyle, 2002). For instance, homeostasis refers to maintaining a steady state, but robustness can also apply to dynamic processes in development. It is important to note that robustness (such as stability) encompasses a relative not an absolute property because no system can maintain stability for all its functions when encountering any kind of perturbation. Hence, we have to specify (1) which characteristic behavior remains unchanged and (2) for which type of disturbances or uncertainties this invariance property holds.

In engineering and biology, the characteristic behavior can be interpreted as a "desired system characteristics" or "performance" (Carlson and Doyle, 2002), thereby directly connecting robustness and functionality. In relatively simple systems, robustness is often equivalent to a dynamical regime. Investigations of genetic oscillators may thus focus on the persistence of a regular periodic solution, which does not preclude quantitative changes (in period or amplitude of the oscillations) to occur (Barkai and Leibler, 1997). Delineating the key function of a particular cellular subsystem, however, is not trivial (Morohashi et al., 2002). Even key inputs and outputs may not be evident in complex networks. The circadian clock, for example, has to provide stable oscillations, but it is unclear whether period, amplitude, phase, or entrainment by light determine the function for the organism (Allada, 2003). More complications arise in cellular information processing, which needs sensitive and robust detection, amplification, and decoding of input signals (Freeman, 2000). Hence, the claim of higher order behavior to be robust and thereby to confer functional advantage requires careful justification.

Cells face uncertainties in the form of externally induced perturbations owing to variable environments (modified inputs) and of internal perturbations such as mutations that affect or entirely remove components and interactions (changed system parameters). Measuring the robustness of a system means to determine the behavior (the output or performance) as a function of the input (the disturbance) (Savageau, 1971; Ma and Iglesias, 2002). A mathematical model thereby serves as a mapping from input space to output space. Focusing on the effects of perturbations in kinetic parameters, Figure 1 shows this dependency for a simplified model of the circadian oscillator in *Drosophila* (Leloup and Goldbeter, 1998). For instance, changes in the two parameters (maximal transcription rates) considered may alter the oscillator's period or completely abolish oscillations. The size of the oscillatory region can serve as a measure of robustness (Ma and Iglesias, 2002; Morohashi et al., 2002). However, as the example demonstrates, the resulting behavior depends on the direction of perturbations, indicating complex synergistic or antagonistic interactions between the parameters.

Another important source of uncertainties are stochastic fluctuations resulting from the random character of biochemical reactions and low copy numbers of
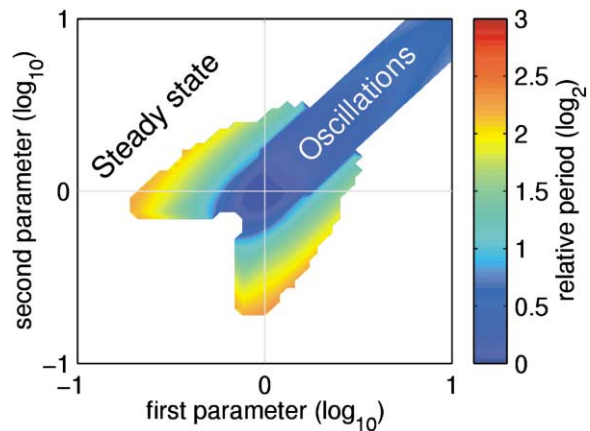

Figure 1. Mapping from Parameter Space to Behavior Space

Here, we calculated the relative period of oscillations for a simplified model of the circadian clock in *Drosophila* (Leloup and Goldbeter, 1998) depending on two of the model's parameters, namely, the maximal rates for transcription of *per* and *tim*. Gray lines indicate the reference point regarding the parameter values, at which oscillations have a period of approximately 24 hr. In the white region, no oscillations occur.

chemical species involved (McAdams and Arkin, 1997). Noise in gene expression with potential amplification and propagation by regulatory dynamics can induce phenotypic variation (Thattai and van Oudenaarden, 2001; Elowitz et al., 2002; Blake et al., 2003). For instance, autoregulation (Becskei and Serrano, 2000; Thattai and van Oudenaarden, 2001) and "stochastic focusing" (similar to the repression of output noise by signal noise in control engineering) (Paulsson et al., 2000) contribute to resistance against noise. Cells, however, seem to have evolved mechanisms to control noise for avoiding detrimental effects (Fraser et al., 2004) as well as for exploiting noise to generate beneficial diversity (Raser and O'Shea, 2004). Again, this underlines that the analysis of robustness in complex systems requires detailed specifications of functions, disturbances, and robustness measures considered.

Robustness, Complexity, and Design
In both biology and advanced technology, the primary function of a system is usually robust to a wide range of perturbations, yet these systems can show extreme fragility toward other (even seemingly much smaller) perturbations and/or other functions. This coexistence of extremes in robustness and fragility ("robust yet fragile") perhaps constitutes the most salient feature of highly evolved or designed complexity. Human-designed technology has well-understood mechanisms, which are deliberately hidden from the user. In contrast, we have little systems level understanding of biological complexity. Here, we argue that by combining the fragmented yet complementary knowledge in both domains, robustness and its associated tradeoffs offer a powerful perspective on biological complexity.

The concept of "*highly optimized tolerance*" (HOT) points out that fixed and limited resources, which are characteristic of technology and biology, call for important robustness tradeoffs. For instance, even minimal

constraints can completely change the "emergent" phenomena of extremely simplified models (Carlson and Doyle, 1999, 2000). The tradeoffs result from robustness (tolerance) being in some sense a conserved quantity, and in particular cases even hard robustness "conservation laws" exist (Csete and Doyle, 2002). More generally, making one feature robust to a class of perturbations makes the same or other features fragile to that or other perturbations. These tradeoffs, however, can be quite subtle even in simple systems. Hence, in design or evolution, robustness, which is adapted to the intended function of a system and the associated uncertainties, must be carefully distributed. HOT therefore emphasizes a necessary connection between complexity and robustness. For instance, cellular complexity appears to arise mainly from robustness as a design goal (Hartwell et al., 1999; Lauffenburger, 2000). Shielding certain functions of a system may require additional control loops; this shielding leads to higher complexity and new potential sources of fragility (Carlson and Doyle, 2002).

Cancer offers an intuitive illustration for these concepts. Under "normal operation," our cells' intracellular mechanisms provide a certain robustness of their behavior (which includes a variety of functions and demands) to diverse perturbations. Cells as components of an organism (network) collectively reduce the impact of environmental perturbations. Thus, the "collective of cells" inherits some of the cells' robustness, augmenting it by synergistic network level interactions. However, cellular fragility can produce robustness at the network level, and increasing robustness from the cells' perspective may be highly detrimental to the organism. Apoptosis of cells carrying potentially damaging, e.g., oncogenic, mutations is an example for the former, whereas uncontrolled, robust growth of neoplastic cells is an example for the latter. Cancer occurs when a cell develops a new behavior that—from its own perspective—is very robust (e.g., against prohibitive signals from the organism). The causative perturbations may be physically small and localized but require large and "complex" changes in information. Viral oncogenes that hijack a single cell's robustness mechanisms are a particularly potent example. More generally, the "rare" nature of fragility reinforces the need to address stochastic events. The network in turn may not be robust to rare behaviors that are abnormal but active. Cancer cells thereby can create a function that kills the organism, its ultimate fragility. Thus, cancer is a multilayered example of "robust yet fragile."

More specifically for the cellular level we focus on, model-based scenarios of gene network evolution in changing environments, in which robust behavior was required, favored simple expression patterns and temporally stable, highly organized structures (Bornholdt, 2001). In experimental studies, independently evolved strains of *E. coli* showed convergent patterns of development toward higher fitness (Cooper et al., 2003). Finally, robustness may in fact not impose barriers to evolution but instead enhance "evolvability" because robustness enables genotypic variability without immediate functional consequences, shortening the path to favorable phenotypes (Kirschner and Gerhart, 1998; de Visser et al., 2003). Hence, although many specific aspects of the implicit robustness tradeoffs are open, we

argue that general design principles exist—with the appropriate caution about general principles and simple stories of complexity and robustness. We therefore focus on a common set of basic mechanisms that confer robustness to biological and engineered systems alike.

Redundancy

The simplest strategy to protect against failure of a specific component is to provide for alternative ways to carry out the function that the component performs. At the molecular level, this backup strategy or "genetic buffering" (Hartman et al., 2001) may be brought about by duplicate genes with identical roles or by different genes that constitute alternative but functionally overlapping pathways. In contrast to redundant systems in engineering, however, identical genes that do not diverge in functionality or regulation would not survive in evolution (Krakauer and Plotkin, 2002). Instead, structurally different entities perform overlapping functions, which lead to the suggestion to use "degeneracy" as a more appropriate term (Edelman and Gally, 2001).

Closer investigation of robustness in cellular networks resulting from redundant genes or pathways, thus, offers one approach to uncover design principles. In this respect, many studies suffer from the fact that direct links between robustness and gene or pathway function were not established. These approaches relied on indirect evidence such as sequence similarity or topological proximity owing to unspecified interactions. Graph theory, for instance, suggests that robustness is a feature only of specific classes of redundant networks (Albert et al., 2000), into which cellular networks do not per se have to belong.

Feedback Control

Control circuits play a decisive role in maintaining cellular functions in the face of internal or external uncertainties. In brief, by using the output of a function to be controlled in order to determine appropriate input signals, feedback enables a system to regulate the output by monitoring it (Figure 2). Negative feedback can reduce the difference between actual output and a given set point, thereby dampening noise and rejecting perturbations. Model-based design and engineering of small artificial genetic circuits provides direct means for testing the predicted features of regulatory schemes in vivo (Hasty et al., 2002). For instance, negative autoregulation of a transcription factor stabilized its steady-state expression levels despite the inherent noise in transcription. Autoregulation proved advantageous for a range of biologically plausible parameters, and high gain of the feedback loop enhanced the effect (Becskei and Serrano, 2000). This work confirmed predictions already made in a classical theoretical study of stability in gene regulation (Savageau, 1974). Similarly, robustness of an oscillatory circuit constructed from three transcriptional repressors (Elowitz and Leibler, 2000) was revealed.

Positive feedback (or autocatalysis), in general, enhances sensitivity. This is primarily required for robust cellular decisions that need to be derived from noisy and graded input signals and to be maintained (Angeli et al., 2004). Consequently, engineered switches resembling units for cellular decision making relied on positive
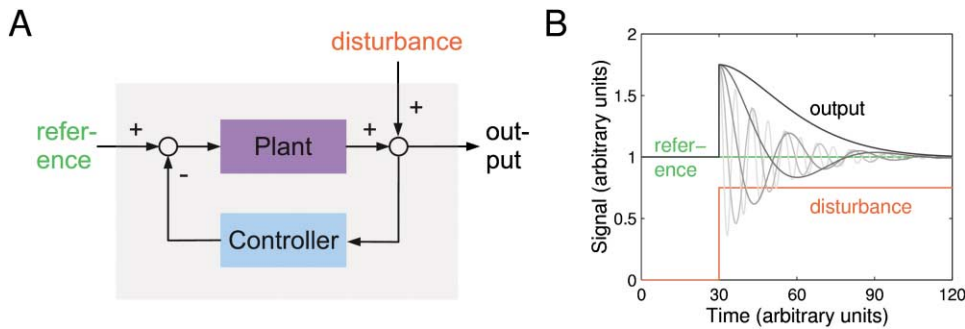
Figure 2. Control by Negative Feedback

(A) General scheme of negative feedback showing a plant (cellular function that is to be controlled) and a controller (regulatory circuit) that feeds back to the plant's input. We consider integral feedback control, where the output signal has to track a reference signal despite perturbations. (B) Rejection of a disturbance when the gain of the feedback loop is varied from low (black) to high (light gray).

feedback (Gardner et al., 2000; Becskei et al., 2001). Depending on the desired functionalities, thus, both forms of feedback and combinations thereof can convey robustness (Freeman, 2000). However, it is essential to note that the role of feedback involves hard tradeoffs, resulting from a "law" on the conservation of robustness (Csete and Doyle, 2002). Feedback introduces fragilities such as the possibility of self-sustaining and cascading failures induced by positive feedback, for instance, in uncontrolled tumor growth (Kitano, 2004). A high gain in negative feedback leads to steady-state stability, but fragilities enter in potentially inaccurate transient responses (Figure 2) because time-varying perturbations can be amplified (Csete and Doyle, 2002).

Thus, the degree to which a control circuit contributes to robustness strongly depends on the circuit design, the control objective, and the type of perturbations affecting the system. Well-balanced positive and negative feedback can lead to a blend of sensitivity and stability. Another possibility for achieving higher robustness consists in combining multiple levels of regulation, for instance, controlled transcription, translation, posttranslational modification, and degradation. Often when highly precise and reliable behavior is indispensable for overall cellular functionality, multiple intertwined feedback loops operate. The circadian clock (Cyran et al., 2003) and developmental control circuits (see below) provide good examples for these aspects.

Modularity
A central, increasingly accepted notion is that cells are composed of "functional units" or "modules" (Hartwell et al., 1999; Lauffenburger, 2000; Csete and Doyle, 2002; Nurse, 2003; Han et al., 2004). Modules constitute semiautonomous entities that show dense internal functional connections but looser connections with their environment. Modularity, the encapsulation of functions, can contribute to both robustness of the entire system (by confining damage to separable parts) and to evolvability (by rewiring of modules or by modifications in modules that are unnoticeable from the outside) (Hartwell et al., 1999). Synthetic lethality of yeast genes predominantly acting in the same (intrinsic) or a functionally similar (extrinsic) pathway concurs with a modular system (Hartman et al., 2001). Several groups analyzed network topologies, that is, the (static or dynamic) pattern of components and their interactions. They detected separable clusters in metabolic (Ravasz et al., 2002), protein-protein interaction (Rives and Galitski, 2003), gene regulatory (Segal et al., 2003; Ihmels et al., 2004), and integrated networks (Tanay et al., 2004). Moreover, topological analysis suggested that cellular regulation uses recurrent, simple building blocks ("network motifs") to perform functions such as feedback control (Shen-Orr et al., 2002; Milo et al., 2002; Lee et al., 2002; Yeger-Lotem et al., 2004). Differences between the extracted modules and canonical pathways (Ravasz et al., 2002; Rives and Galitski, 2003; Ihmels et al., 2004) may be explained by more flexible concepts of modularity, which allow one component to occur in several units (Hartwell et al., 1999) or participate dynamically in the formation of modules (Han et al., 2004).

In many domains besides biology, it has been proposed that encapsulation of simpler functions in modules can reduce the risk of catastrophic failure by preventing that damage in one part spreads throughout the network (Hartwell et al., 1999; Albert et al., 2000). Many cellular networks are claimed to have a "scale-free" organization with few central hubs and many nodes bearing few links (Barabási and Oltvai, 2004). Such structures were suggested to resist random perturbations because of a low probability that a perturbation affects the sensitive hubs (Albert et al., 2000; Jeong et al., 2000; Ravasz et al., 2002). However, it is unclear to what extent these high-level abstractions are able to capture network functionality because experimental observations barely support key predictions of the theory (Hahn et al., 2004). If, for instance, crosstalk was vital for coordination of cellular functions, the pure topological argument implying equal importance of all interactions would not hold. Moreover, robustness of modules is distinct from robustness of collections of modules such as pathways or cells. Thus, we will have to account for the interplay between different cellular networks, their potential dynamics, and foremost, a rigorous comparison with experimental observations.

Hierarchies and Protocols
Protocols encompass the set of rules underlying the efficient management of relationships between the parts

(i.e., modules) that constitute a system. They include the organizational structures for embedding modules and the interfaces between modules that allow for system function. Feedback, for instance, is a particular protocol. General currencies for energy and reduction equivalents in metabolism and shared mechanisms of gene expression, covalent modifications, or controlled degradation in regulation provide common standards for interactions between the parts (Csete and Doyle, 2002). Protocols, hence, are of primary importance for an understanding of how information in living cells is integrated (Hartwell et al., 1999), but they have received little attention in the context of cellular robustness.

As one particular type of protocol, an efficient means for coordination in complex systems is to organize a system hierarchically, namely to establish different layers of integration to reduce the costs of information transmission (Guimerà et al., 2001). Cellular control constrains the system's possible behavior on a lower level by regulation at higher levels. In transcriptional regulation, the presence of RNA polymerase offers a wide variety of different gene expression patterns, controlled by regulation recruitment (Ptashne and Gann, 1997). Combinatorial control via associated factors and specific transcription factors adjusts the actual transcription rate. This enables the cell to establish global to local layers of regulation by controlling the availability of increasingly specific components (Holstege et al., 1998). In bacterial cell cycle regulation, for instance, genome-wide analysis of gene expression revealed such a hierarchical control architecture (Laub et al., 2000). Similar themes appear in translation and intracellular proteolysis. This separation of functions, and their integration at higher levels, could constrain the effects of local deregulation and provide common standards for robust coordination of cellular functions (Csete and Doyle, 2002; Ravasz et al., 2002). A major challenge thus consists in elucidating the links between different levels of organization to see how protocols and hierarchies handle the tradeoffs between robustness and efficiency (Stelling et al., 2002, 2004).

Bacterial Chemotaxis
Bacterial chemotaxis, in particular in *E. coli*, is one of the best-studied model systems for signal transduction. The relative simplicity of the chemotaxis system contributed to its selection. The network (Figure 3) encompasses transmembrane receptors, the adaptor CheW, a two-component signaling system (CheA/CheY), the output of which controls the motor's tumbling frequency, and CheR/CheB that modify CheA's activity via the receptor's methylation state (Hazelbauer et al., 1993). Despite the system's apparent simplicity, it shows features of advanced biological regulation such as high sensitivity to environmental changes over a broad dynamic range, adaptation to constant stimuli enabling its operation in a noisy environment, and robustness (Alon et al., 1999).

In a pioneering theoretical study, Barkai and Leibler (1997) suggested that specifically the property of perfect adaptation (but not, for instance, the adaptation time) is robust to variation in the components' biochemical properties. In their simplified biochemical model, feed-

back at the level of receptor methylation conferred robustness, which later was confirmed experimentally (Alon et al., 1999). A control perspective on this model finally indicated that bacteria have adopted the standard engineering trick of integral feedback control: the time integral of the difference between actual output and set point is fed back into the system via the methylation state of the receptor (Yi et al., 2000). As integral feedback is necessary and sufficient for robust steady-state control, it explains robustness of phenomena such as perfect adaptation, homeostasis, and noise rejection (Csete and Doyle, 2002). Hence, identifying equivalent control schemes in biology and engineering may be a promising path to understanding cellular complexity.

However, control at the receptor level is by far not sufficient to understand bacterial chemotaxis. Long-range interactions between receptors (Sourjik and Berg, 2004) as well as the control of the flagellar motor by phosphorylated CheY (Cluzel et al., 2000) are highly cooperative, causing a high gain of the signal transduction pathway. Concomitantly, the steady-state concentration of CheY-P must be maintained at the most sensitive region of the motor response curve, while *E. coli* to a certain extent seems to use molecular noise to induce behavioral variability for optimizing its search for nutrients (Korobkova et al., 2004). Multistage feedback regulation downstream of the receptor complex that involves CheA/CheZ and impinges on CheY could be a key to the overall robustness of chemotaxis (Almogy et al., 2001). We anticipate that such intertwined feedback loops will be of even greater importance for understanding more complex networks. A model-based comparison of chemotaxis in *E. coli* and *B. subtilis* illustrates potential benefits of such an approach. While the network topology, albeit constructed from similar proteins, is quite different (Figure 3), the analysis revealed a conservation of the "core logic." Moreover, additional feedback loops in *B. subtilis* make specific features of the system more robust (Rao et al., 2004), underlining the connection between robustness and (regulatory) complexity. Hence, chemotaxis is an excellent example for nested feedback as a means to achieve robustness, and it underlines individual roles and significances that different instances of feedback possess in cellular regulation (Freeman, 2000).

Microbial Metabolic Networks
By interconnecting hundreds of intracellular metabolites through extensive networks of biochemical reactions, cellular metabolism ensures appropriate supply of all cellular components, energy, and cofactors from a wide range of nutrients. Global organization and properties of metabolic networks (Almaas et al., 2004; Covert et al., 2004; Stelling et al., 2002) become tractable through their large-scale in silico reconstruction (Papin et al., 2004) and experimental methods for functional analysis of network operation (Sauer, 2004). Perhaps the most astounding property of microbial metabolism is its evolved robustness to sustain survival and proliferation upon extensive environmental or genetic perturbations.

Robust growth under ever-changing environmental conditions is mostly ensured by genetic and enzyme-level regulation, which modifies the availability and effi-
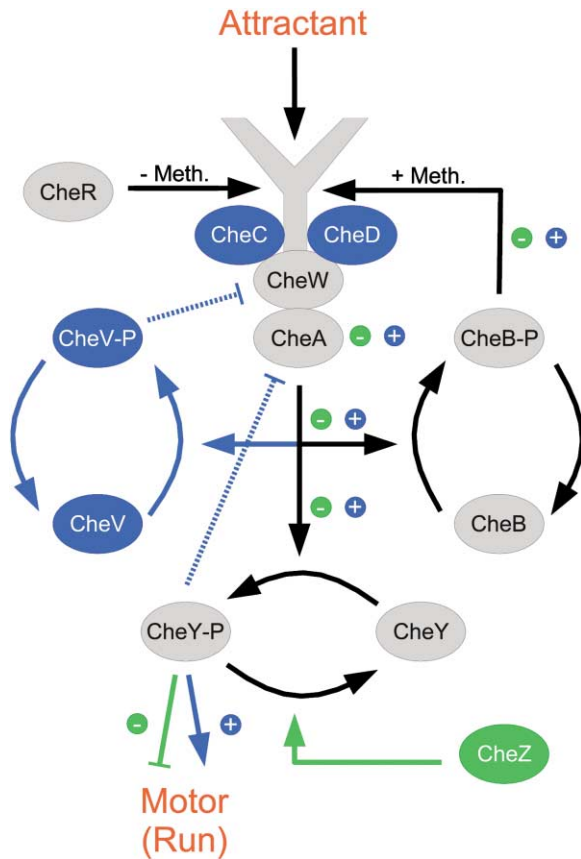
ciency of connections between network components (Ihmels et al., 2003). The two underlying principles of environmental robustness are modularity and hierarchical organization. Spatially (e. g. organelles) but mostly chemically (e.g., particular degradation or biosynthesis pathways) isolated functional modules that carry discrete functions are controlled in a hierarchical manner that restricts module operation to the appropriate conditions. On top of the hierarchical cascade are multitarget regulation mechanisms such as, for example, carbon catabolite repression, which controls expression of degradation pathways for a large number of alternative but less preferred substrates (Warner and Lolkema, 2003). Induction/repression of individual pathways by their cognate substrate/product is typically at the lower end of the regulation cascade.

Buffering of minor heritable genetic variation within a population such as single-nucleotide polymorphism is a key element in understanding human disease (Hartman et al., 2001). In haploid microbes, this buffering becomes mostly apparent as genetic robustness against null mutations with many dispensable genes under a given condition (Gerdes et al., 2003; Giaever et al., 2002). The two primary mechanisms that bring such robustness about are redundancy of components (genetic buffering) and modules with overlapping functions (functional complementation) (Gu, 2003). For metabolism, it has been estimated that about one quarter of those gene deletions that have no apparent phenotype are compensated by genetic buffering with duplicate genes and significantly less by functional complementation through

flux redirection (Papp et al., 2004). The remaining fraction of 37%–68% was simply not relevant under the conditions investigated.

Generally, genetic buffering and functional complementation are not equally distributed. Most peripheral anabolic pathways are essential unless their product is supplied externally (e.g., amino acids). Thus, knockouts are lethal with some genetic robustness from few duplicate genes. Peripheral catabolic pathways, in contrast, are often redundant with multiple or broad specificity enzymes and transporters, with the 20 hexose transporters of *Saccharomyces cerevisiae* as a particularly extreme example (Ozcan and Johnston, 1999). This difference reflects the cell's ecological challenge to synthesize a well-defined set of cellular components from whatever nutrients are available. All peripheral catabolic fluxes finally merge into the interconnected network of central metabolism that catalyzes the most massive fluxes that distribute the material flow into biosynthesis, energy generation, or waste. While extensive duplicate genes contribute to genetic robustness of some microbes like *S. cerevisiae*, mostly the modular structure confers significant robustness to central metabolism. A prominent example of biochemically and genetically distinct but functionally redundant pathways are the two main routes of glucose metabolism, glycolysis and the pentose phosphate pathway, that can often substitute for each other (Sauer et al., 2004). In some cases, the exact molecular cause for robustness has been elucidated by quantifying the flux redistribution in knockout mutants, as illustrated by the local flux rerouting in *Escherichia coli* pyruvate
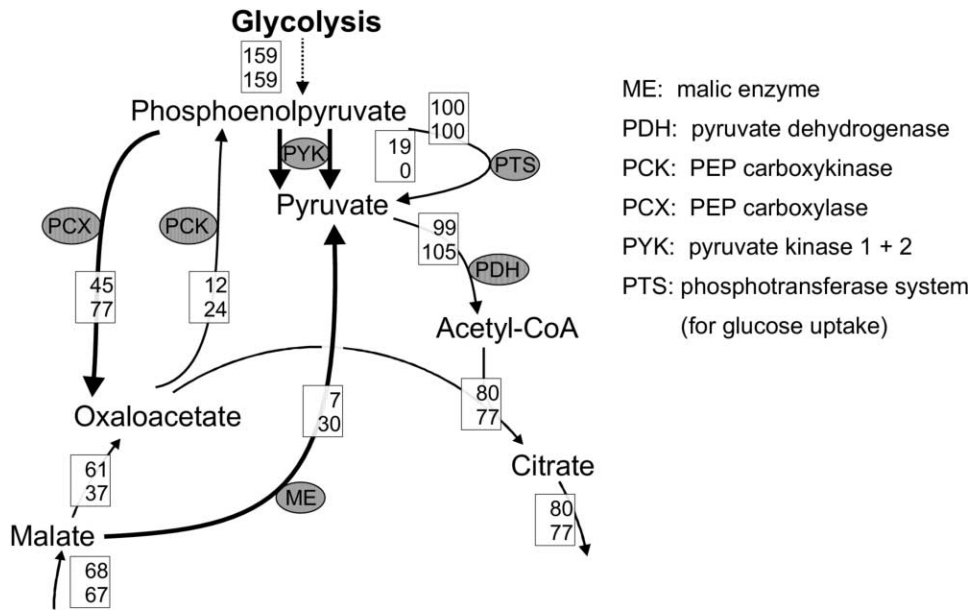
Figure 4. Distribution of Metabolic Fluxes at the Glycolysis-Krebs Cycle Interface in *E. coli*

Top entries in the boxes denote fluxes in wild-type, whereas bottom entries provide the values for a knockout mutant of both pyruvate and kinase isoenzymes. Flux values are relative to the specific glucose uptake rate and were obtained from glucose-limited continuous cultures at a growth rate of 0.4 h^{-1} (Emmerling et al., 2002). The local bypass of pyruvate kinase via malic enzyme and PEP carboxylase is highlighted with bold arrows.

kinase mutants with an otherwise unaltered phenotype (Figure 4).

Lastly, metabolic network operation is relatively robust to stochastic fluctuations. Despite natural variations in the absolute concentrations of metabolites and enzymes within individual cells of a population or in independent experiments, the physiological phenotype and the distribution of intracellular fluxes remain rather stable (Fischer and Sauer, 2003). As a corollary of such stochastic robustness, manipulation of individual enzyme concentrations within a metabolic pathway rarely affects the molecular flux through the pathway, and thus the phenotype, provided the enzymes follow Michaelis-Menten kinetics and are not saturated with substrate as is typically the case (Kacser and Burns, 1973). The consequence of this stochastic robustness is "appreciated" as network resilience to genetic manipulations in metabolic engineering of cellular functions (Bailey, 1999). The molecular basis is primarily feedback control, where the distributed kinetic properties of all enzymes in a pathway buffer natural noise in the pathway components. Finally, the single robustness mechanisms operate in a highly organized, overall architecture. Cooperation is an essential aspect of it because, for instance, switching between redundant pathways requires some sort of feedback. The architecture might thus allow highly optimized tradeoffs between different objectives, one of which is robustness (Csete and Doyle, 2004).

Developmental Control Circuits

The development of multicellular organisms is in many ways the most studied and appreciated example of robust networks. Despite a wide range of experimental and "real life" perturbations, the developmental program

often leads to the same phenotype of the mature organism. The analysis of developmental control networks, however, involves an additional layer of complexity: while, for example, microbial metabolic networks are limited to the inner environment of a single cell, developmental control circuits involve a rich network of intercellular communication as well. Detailed analysis of the segment polarity network of *Drosophila* highlights how the principle of robustness can be applied to studying multicellular systems.

Segmentation is a key step during embryonic development (DiNardo et al., 1994). Layers of cells become committed to various differentiation fates by the exclusive activation of genes such as *wingless* or *engrained* (DiNardo et al., 1994). The activation of these genes follows a highly coordinated spatial distribution with sharp boundaries between expressing and nonexpressing cell layers. The sharp spatial (and often temporal) gradients imply a high level of robust regulation, which is of great research interest. Molecular biology has mapped out a significant portion of the topology of the segment polarity network, but reliable kinetic parameters could be determined with much less certainty (von Dassow et al., 2000). Therefore, the network topology cannot be turned directly into a dynamic model, which is usually the starting point to understanding dynamic pattern formation. At this juncture, von Dassow et al. (2000) turned to robustness as an overall simplifying criterion. This practice is somewhat reminiscent of the use of conservation laws in physics: certain end states can be correctly predicted even if the intervening dynamic states cannot be precisely described. In this case, if the network topology is correct and ensures robust behavior, then random sets of kinetic parameters should often produce the required spa-

tial distribution of gene expression patterns. Von Dassow et al. (2000) found that for robust behavior in terms of resistance to changes of kinetic parameters within up to a 1000-fold range, they needed to add two more regulatory interactions, completing two positive feedback loops. Circumstantial evidence supported the existence of these regulatory interactions. However, the topology of the network required corrections because the model did not maintain robustness under certain conditions such as cell division. Consequently, several changes were introduced, most notably the inclusion of the *sloppy pair* gene (Albert and Othmer, 2003). This updated topology produced robust patterning in a highly simplified model under a wide variety of conditions including cell divisions and gene mutations (Albert and Othmer, 2003). In a more detailed analysis, Ingolia (2004) demonstrated that most of the behavior of the segment polarity network model could be understood in terms of bistability generated by positive feedback of gene expression in individual cells. In particular, random parameter sets that did not conform to the rules ensuring bistability almost never produced the required pattern, whereas half of the parameter sets satisfying this criterion generated it. This was not completely unexpected because of the all or none type expression of genes, the prepattern as a prerequisite for robust gene expression, and our knowledge on a significant number of sharp switches in biology governed by bistable positive feedback loops (Pomerening et al., 2003; Sha et al., 2003; Lai et al., 2004).

The increasingly detailed investigation of the segment polarity network, therefore, suggests that modularity, positive feedback loops, common protocols, and the hierarchical organization of gene expression regulation by transcription factors are necessary and sufficient to ensure the robustness of this particular regulatory network. Whether other developmental regulatory networks resist such decomposition remains to be seen, but we cannot exclude the possibility that nature employs more complex design principles than the bottom-up assembly of human engineering.

Robustness as a Research Tool

The study by von Dassow et al. (2000) exemplifies how the principle of robustness could be exploited for studying dynamic networks when kinetic parameters are largely unknown. A more pointed scientific strategy, where the robustness criterion is used to produce testable hypotheses, relies on the following logic: biological systems are robust—therefore an appropriate mathematical representation must also be robust. Consequently, mechanistic details that are necessary to satisfy this criterion form testable hypotheses. This approach is best exemplified by a study on the mechanistic basis of the robust BMP morphogen gradient in *Drosophila* embryo development. Eldar et al. (2002) created an ordinary differential equation-based model that included only a few regulatory proteins known to play a key role in this system. The relative simplicity of the model allowed the creation and meaningful analysis of a large number of model networks based on random parameter sets. Only a subset of all model networks, less than one percent, displayed robust behavior. Remarkably, robust networks

shared certain parameter properties that were not present in the non-robust models. Furthermore, these shared properties could be readily translated into actual biological concepts, such as the diffusion properties of a given protein. Several such concrete biological hypotheses were then tested and validated by direct biological experimentation.

Bottom-up or top-down modeling of cellular regulatory subnetworks has been attempted both by academic groups and commercial ventures (Schoeberl et al., 2002; Kansal, 2004; Christopher et al., 2004). This requires the careful and detailed coupling of individual biological components, such as protein concentrations, by dynamic interactions. Although, the upfront knowledge-based effort is obviously significant, it also ensures its appeal to biologists: the dynamic interactions driving the model are based on established biological knowledge. However, quantitative modeling approaches require the identification of a significant number of kinetic parameters, mostly by minimizing the deviations between measurements and model predictions. Currently, this is the major bottleneck in model development. For instance, the identification methods can be rather misleading for certain networks and overfitting often occurs when the ratio of parameters to be optimized relative to high-quality experimental information is unfavorable. However, as the analysis of developmental circuits indicates, a correct network topology ensuring robustness may be the crucial information needed. In this way, relevant dynamic models can be created mainly based on topology without the necessity of accurate parameter optimization, thus avoiding the well-known artifacts associated with such approaches. Thus, "topology driven' robustness may provide the remedy to the numerous ailments affecting large-scale dynamic modeling of biological networks.

Conclusions

The view that biological systems are highly resistant to perturbations is largely undisputed, while no generally accepted approach to the analysis of robustness in cellular systems exists. In any instance, however, one has to take into account that robustness refers to the maintenance of specific functionalities of a system subjected to specific perturbations. The current knowledge about robustness in biological systems, and the cellular design principles underlying it, shows a large bias. At very high levels of abstraction, concepts such as highly optimized tolerance elaborate general features, namely the trade-off between efficiency and robustness and necessary connections between robustness and complexity. Large-scale topological analysis gives clues on structural features that could confer robustness to cellular function. Both high-level approaches, however, currently suffer from the fact that their central hypotheses remain to be evaluated by analyzing specific biological examples through mathematical modeling and experimental observation. At the other end of the spectrum, studies of robustness in small systems proved successful. Their extension to more complex systems may enable one to infer general design principles.

We argue that future investigations should be guided by and specifically address current hypotheses on a

common set of mechanisms that contribute to robustness in biology and engineering. The relative importance of redundancy of components versus pathways, the role of individual feedback circuits, the modular organization of cellular networks, and the integration of cellular functionality across hierarchies could be of predominant interest. The notion of cells being composed of robust subunits of limited autonomy simplifies modeling and abstraction of general properties. We can proceed from the detailed investigation of individual modules to their interplay and its consequence for overall systems performance. Robustness of cellular systems, hence, provides us with testable hypotheses derived from top-down studies and with opportunities for a more detailed bottom-up approach. Both approaches should finally converge.

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