Hypothesis

Robustness, evolvability, and neutrality

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Abstract **Biological systems, from macromolecules to whole organisms, are robust if they continue to function, survive, or reproduce when faced with mutations, environmental change, and internal noise. I focus here on biological systems that are robust to mutations and ask whether such systems are more or less evolvable, in the sense that they can acquire novel properties. The more robust a system is, the more mutations in it are neutral, that is, without phenotypic effect. I argue here that such neutral change – and thus robustness – can be a key to future evolutionary innovation, if one accepts that neutrality is not an essential feature of a mutation. That is, a once neutral mutation may cause phenotypic effects in a changed environment or genetic background. I argue that most, if not all, neutral mutations are of this sort, and that the essentialist notion of neutrality should be abandoned. This perspective reconciles two opposing views on the forces dominating organismal evolution, natural selection and random drift: neutral mutations occur and are especially abundant in robust systems, but they do not remain neutral indefinitely, and eventually become visible to natural selection, where some of them lead to evolutionary innovations.**
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1. Introduction

The word evolvability has two main usages [1–4]. 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.

These definitions apply to biological systems on all levels of biological organization, such as macromolecules like RNA and proteins, metabolic pathways, gene regulation networks, macroscopic traits, and whole organisms. In consequence, functional innovation also comes in many different sizes and shapes, from enzymes with new catalytic activities, to novel complex organs such as eyes or wings [5].

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.

Living things are unimaginably complex, yet also highly robust to genetic change on all levels of organization. Proteins can tolerate thousands of amino acid changes, metabolic networks can 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 genetic change in embryonic development can lead to an essentially unchanged adult organism [6–9]. Such robustness is one of several factors that can affect evolvability in either sense [1]. My central question here is whether robustness fosters or hinders evolvability. Clearly, 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 problem, and my focus in this article.

One can adopt two conflicting perspectives on this problem. The first arises from the observation that robustness causes many mutations to be neutral, mutations with no phenotypic effect on the system. 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, in contrast, gives neutral mutations a key role in innovation: although many

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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 to fulfill 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 [10] 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.

2. Neutrality, can it be assessed experimentally?

A key difference between the two perspectives of the last two paragraphs is their tacit understanding of neutrality. I will now examine this notion more closely. Neutral genetic change, made prominent by Kimura [11] in his neutral theory of molecular evolution, 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.

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 [12]. 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 in practice not exist.

A second candidate approach to identify neutral mutations applies to well-understood systems inside an organism. For example, assume 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 [13]. In vertebrates, it is the same protein as neuroleukin, a cytokine causing immune cell maturation, and survival of some embryonic spinal nerve cells [14,15]. In addition, phosphoglucose isomerase also serves as autocrine motility factor [16], a cytokine that stimulates cell migration. As if that were not enough, it can also cause differentiation of human myeloid leukemia cells [17]. 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, 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 [18]. Among a long list of further examples [13] is thymidine phosphorylase, which catalyzes the dephosphorylation of thymidine and deoxyuridine, and is the same as an endothelial growth factor [19,20].

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 [21,22]. Taken together, all these examples show that measuring 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. These examples also show that we can never be sure that all the right questions have been asked.

3. The evolutionary approach to identifying neutrality

The last paragraphs show that neither fitness nor a biological system's performance – that is, all conceivable aspects of it – can be measured in practice. If so, one might think that the above definition of (fitness-centered) neutrality is operationally useless. However, experimentation is not the only way to ascertain the neutrality of mutations. The alternative is an evolutionary approach that 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. I will briefly discuss this approach and ask whether it can rescue the essentialist concept of neutrality. The approach takes advantage of a simple yet fundamental population genetic insight: Neutral mutations that occur in a population go to fixation (they attain a population frequency of one) at a clock-like and constant rate [11]. Importantly, this rate depends only on the rate at which neutral mutations occur, and not on other factors, such as population size. The rate is thus independent of the peculiarities of a population's demographic history. This does not apply to mutations subject to natural selection, whose fate is influenced by such factors.

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 [23]. 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 when viewed from this evolutionary perspective? 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 30 years of debate and data analysis, the selectionists would clearly win by points [24–26]. One of the key insights that emerged from the neutralist–selectionist debate is that even the most obvious candidates for neutral mutations have provided evidence for selection. Among the best examples are mutations in a gene that change one codon into another codon for the same amino acid. Such synonymous mutations are paradigmatic candidate examples of neutral mutations. Yet such mutations can reduce the rate at which a messenger RNA is translated into protein, if they occur towards 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 [27–29]. In addition, if a gene's optimal expression level changes over time, then the strength of selection on its synonymous mutations may also change.

It is easy to conceive of potential examples for neutral mutations other than synonymous mutations. They include mutations in gene-poor parts of the genome, such as telomeric regions and heterochromatin, or mutations in non-coding and non-regulatory 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 non-coding human heterochromatin, perhaps in a sequence that is a member of the *Alu*-family of short repetitive interspersed elements [30]. Such mutations are classical candidate example of neutral mutations, mutations in 'junk' DNA. However, because genome rearrangements large and small are frequent in many eukaryotes [31], 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 non-coding 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 will just cite examples from 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 [32]. More generally, many metabolic genes that are dispensable in one environment may be essential in another. 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 [33–35].

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 [36]. The incidence and severity of this disease 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 US (higher altitude) [36]. The population genetic and epidemiological data indicate that at least some alleles associated with this disease can spread through random genetic drift in the Dutch population, but not in the US population, where natural selection is stronger [36]. 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 other 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 [26]: a mutation may affect fitness at some times but not at others.

4. 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 non-existent. 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 biological systems highly robust to mutations? 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, microbes and higher organisms that can tolerate the elimination of many genes, and different cell division and interaction patterns that lead to the formation of the same organ or organism [6–9,37]. 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 non-neutral 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 and relatedly, we must abandon an essentialist notion of neutrality. That is, a once neutral mutation may affect the system's function in a changed environment or genetic background. Because of its importance, I will illustrate this principle with some further examples, the first of which regards RNA molecules.

For many RNA molecules, the right secondary structure is a prerequisite for their function. This is well known for secondary structure elements of messenger RNAs, and for the genomes of RNA viruses [38–46]. It means two things. First,

changing the molecule's structure may affect its function. Second, a mutation's neutrality with respect to secondary structure becomes a worthwhile subject of study. Fig. 1 kindly provided by W. Fontana, illustrates 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 [47]. Specifically, the $C \rightarrow G$ substitution at one particular position – in and by itself 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 [47].

An 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 non-neutral if certain genes, such as the gene encoding the heat shock protein Hsp90, undergo mutation. As a result of such mutations, genetic variation in phenotypic characters such as eyes and wings increases. Natural selection can readily act on this phenotypic variation [48].

Yet another example is provided by "monogenic" diseases, which are diseases typically attributed to mutations in one
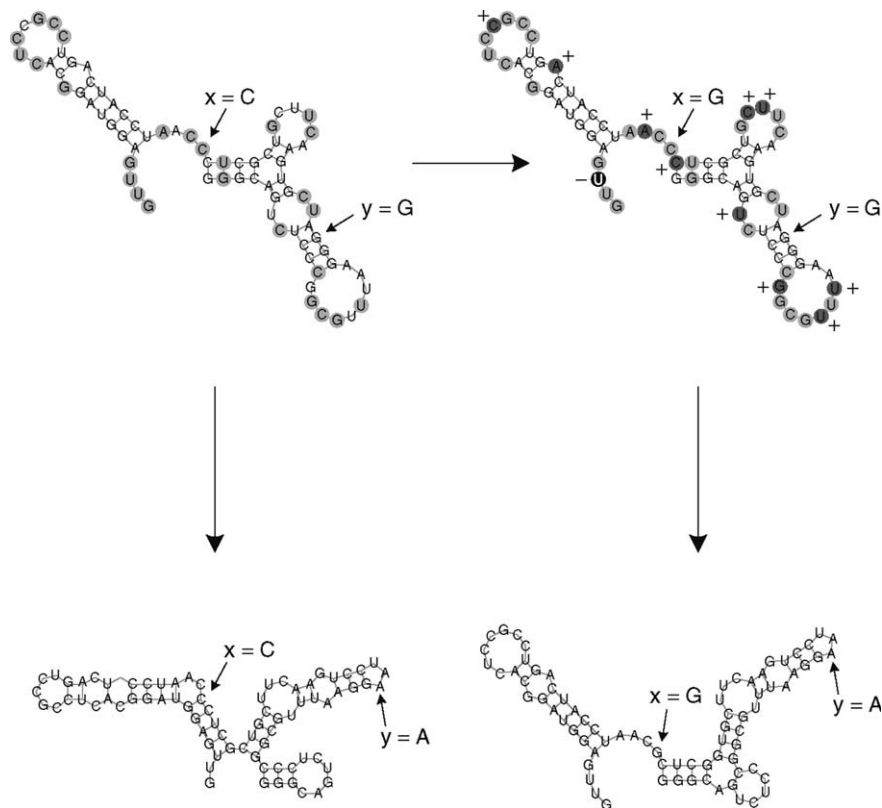

Fig. 1. Neutral changes that make a difference. The figures show the computationally predicted minimum-free energy secondary structure of an RNA molecule. Gray bullets on the upper two secondary structures indicate neutral positions, that is, positions where at least one possible nucleotide change does not alter the structure. In the top left sequence, position x is neutral, because a $C \rightarrow G$ substitution preserves the structure, as shown on the right hand side of the top. However, neutral positions themselves change as a result of this neutral substitution. The dark gray bullets ("+") and the black bullet ("−") indicate positions that have become neutral or stopped being neutral, respectively, as a consequence of this $C \rightarrow G$ substitution. The lower part illustrates that the neutral $C \rightarrow G$ mutation at position x changes the consequences of changing A to G at the (non-neutral) position y, that is, the structural changes this non-neutral $A \rightarrow G$ mutation causes. Redrawn from a figure kindly provided by Walter Fontana [47].

key gene. Increasingly detailed genetic analyses of such diseases show that the notion of one disease-causing genes is vastly oversimplified: for very common genetic diseases such as cystic fibrosis, phenylketonuria, and thalassemia, one and the same mutation may cause severe disease in one individual, and have a lesser or even no effect in another individual [49,50]. The reasons are both genetic and environmental. A final, environmental example is provided by the sensitivity of photosynthesis to the activity of the enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase-oxygenase) in tobacco. This sensitivity varies drastically with the lighting conditions under which plants are grown [51].

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

> A neutral mutation does not change one 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 [52]. 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 non-essentialist concept. The biological species concepts 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 non-essentialist perspective on neutrality? First and most simply, a name, a name for an aspect of a biological system that remains unperturbed in the face of change. 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 time scales of molecular evolution studies, most mutations are not fitness-neutral. Thirdly, like any other concept, the non-essentialist concept of neutrality will have limitations and grey areas where its application is awkward.

5. 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 possible 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. Then, neutrality can become key to innovation.

Many possible examples could be used to illustrate anecdotally how neutral change in this sense could foster innovation [2]. 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 discussed above. They all contain loopholes and are suggestive rather than conclusive. To close these loopholes is a major task of future research in this area.

Computational work on RNA structure shows how repeated mutations, neutral with respect to RNA secondary structure, can explore a space of RNA sequences such that new structures – structural innovations – can become more accessible through single mutations. An important experiment by Schultes and Bartel [53] suggests that a similar principle may apply not only to secondary structure – a 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 converted into each other 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 innovations.

A second class of candidate examples regards the multifunctional proteins for which I mentioned some examples earlier. Multifunctional 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 lead to innovations in these proteins.

A recent laboratory study of enzyme evolution provides a different twist on this question [54]. The study regards the evolution of three different enzymes, each of which has one primary catalytic function and several other, much weaker catalytic activities. One of these enzymes is human serum paraoxonase (PON1). Its primary activity is the hydrolytic cleavage of lactones (cyclic esters). A laboratory evolution experiment shows that this enzyme can readily acquire a massively increased ability to hydrolyze a synthetic organophosphate without greatly changing its primary activity. Similar results hold for the other two enzymes [54].

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 non-duplicated crystallins also exist. They include ε-crystallin, which is the same as lactate dehydrogenase and τ-crystallin, which is the same as α-enolase [55,56]. In such non-duplicated 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 readily give rise to new binding motifs for transcription factors by chance alone [57]. Even in lower eukaryotes such as yeast – which has much smaller regulatory regions than higher eukaryotes – regulatory regions can evolve extremely rapidly.

Candidate examples of neutral change leading to innovation can also be found at the next-higher level of biological organization, that of genetic networks. One case in point is provided by the genes of the segment polarity gene network in the fruit fly *Drosophila melanogaster*. This network is critical to proper segmentation of the fly embryo, and many of its genes have highly conserved function and expression patterns that 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 [22].

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 the root cause of many – although not all [58] – adaptations that distinguish a human from a monkey [59]. It is a sign of how important neutral change, properly defined, can be for innovation.

References

[1] Kirschner, M. and Gerhart, J. (1998) Evolvability. Proceedings of the National Academy of Sciences of the United States of America 95, 8420–8427.

[2] Gerhart, J. and Kirschner, M. (1998) Cells, Embryos, and Evolution, Blackwell, Boston.

[3] Dawkins, R. (1989) in: Artificial Life: The Proceedings of an Interdisciplinary Workshop on the Synthesis and Simulation of Living Systems (Langton, C.G., Ed.), Addison-Wesley, Redwood City, CA.

[4] Poole, A., Phillips, M. and Penny, D. (2003) Prokaryote and eukaryote evolvability. Biosystems 69, 163–185.

[5] Muller, G. and Wagner, G. (1991) Novelty in evolution – restructuring the concept. Annual Review of Ecology and Systematics 22, 229–256.

[6] Rennell, D., Bouvier, S., Hardy, L. and Poteete, A. (1991) Systematic mutation of bacteriophage T4 lysozyme. Journal of Molecular Biology 222, 67–87.

[7] Edwards, J.S. and Palsson, B.O. (2000) Robustness analysis of the *Escherichia coli* metabolic network. Biotechnology Progress 16, 927–939.

[8] von Dassow, G., Meir, E., Munro, E. and Odell, G. (2000) The segment polarity network is a robust development module. Nature 406, 188–192.

[9] Raff, R.A. (1996) The Shape of Life, The University of Chicago Press, Chicago, IL.

[10] Gould, S. and Vrba, E. (1982) Exaptation – a missing term in the science of form. Paleobiology 8, 4–15.

[11] Kimura, M. (1983) The Neutral Theory of Molecular Evolution, Cambridge University Press, Cambridge.

[12] Lewontin, R.C. (2003) Four complications in understanding the evolutionary process. Santa Fe Institute Bulletin 18(online).

[13] Jeffery, C. (1999) Moonlighting proteins. Trends in Biochemical Sciences 24, 8–11.

[14] Chaput, M., Claes, V., Portetelle, D., Cludts, I., Cravador, A., Aburny, A., Gras, H. and Tartar, A. (1988) The neurotrophic factor neuroleukin is 90 percent homologous with phosphohexose isomerase. Nature 332, 454–455.

[15] Faik, P., Walker, J., Redmill, A. and Morgan, M. (1988) Mouse glucose-6-phosphate isomerase and neuroleukin have identical 3′ sequences. Nature 332, 455–456.

[16] Watanabe, H., Takehana, K., Date, M., Shinozaki, T. and Raz, A. (1996) Tumor cell autocrine motility factor is the neuroleukin/phosphohexose isomerase polypeptide. Cancer Research 56, 2960–2963.

[17] Xu, W., Seiter, K., Feldman, E., Ahmed, T. and Chiao, J. (1996) The differentiation and maturation mediator for human myeloid leukemia cells shares homology with neuroleukin or phosphoglucose isomerase. Blood 87, 4502–4506.

[18] Martinis, S.A., Plateau, P., Cavarelli, J. and Florentz, C. (1999) Aminoacyl-tRNA synthetases: a family of expanding functions. EMBO Journal 18, 4591–4596.

[19] Furukawa, T., Yoshimura, A., Sumizawa, T., Haraguchi, M., Akiyama, S., Fukui, K., Ishizawa, M. and Yamada, Y. (1992) Angiogenic factor. Nature 356, 668.

[20] Haraguchi, M., Miyadera, K., Uemura, K., Sumizawa, T., Furukawa, T., Yamada, K., Akiyama, S. and Yamada, Y. (1994) Angiogenic activity of enzymes. Nature 368, 198.

[21] Gilbert, S.F. (1997) Developmental Biology, Sinauer, Sunderland.

[22] Keys, D., et al. (1999) Recruitment of a hedgehog regulatory circuit in butterfly eyespot evolution. Science 283, 532–534.

[23] Kreitman, M. (2000) Methods to detect selection in populations with applications to the human. Annual Review of Genomics and Human Genetics 1, 539–559.

[24] Kreitman, M. (1996) The neutral theory is dead: long live the neutral theory. Bioessays 18, 678–683.

[25] Kreitman, M. and Akashi, H. (1995) Molecular evidence for natural selection. Annual Review of Ecology and Systematics 26, 403–422.

[26] Eanes, W.F. (1999) Analysis of selection on enzyme polymorphisms. Annual Review of Ecology and Systematics 30, 301–326.

[27] Zeng, L., Comeron, J., Chen, B. and Kreitman, M. (1998) The molecular clock revisited: the rate of synonymous vs. replacement change in *Drosophila*. Genetica 103, 369–382.

[28] Comeron, J., Kreitman, M. and Aguade, M. (1999) Natural selection on synonymous sites is correlated with gene length and recombination in *Drosophila*. Genetics 151, 239–249.

[29] Comeron, J. and Aguade, M. (1996) Synonymous substitutions in the Xdh gene of *Drosophila*: heterogeneous distribution along the coding region. Genetics 144, 1053–1062.

[30] Li, W.-H. (1997) Sinauer, Molecular Evolution, Massachusetts.

[31] Eichler, E. and Sankoff, D. (2003) Structural dynamics of eukaryotic chromosome evolution. Science 301, 793–797.

[32] Hartl, D.L., Dykhuizen, D.E. and Dean, A.M. (1985) Limits of adaptation: the evolution of selective neutrality. Genetics 111, 655–674.

[33] Moran, N.A. and Baumann, P. (2000) Bacterial endosymbionts in animals. Current Opinion in Microbiology 3, 270–275.

[34] Frank, A.C., Amiri, H. and Andersson, S.G.E. (2002) Genome deterioration: loss of repeated sequences and accumulation of junk DNA. Genetica 115, 1–12.

[35] Wixon, J. (2001) Reductive evolution in bacteria: *Buchnera* sp., *Rickettsia prowazekii* and *Mycobacterium leprae*. Comparative and Functional Genomics 2, 44–48.

[36] Astrom, K., Cohen, J.E., Willett-Brozick, J.E., Aston, C.E. and Baysal, B.E. (2003) Altitude is a phenotypic modifier in hereditary paraganglioma type 1: evidence for an oxygen-sensing defect. Human Genetics 113, 228–237.

[37] Wagner, A. (2000) Mutational robustness in genetic networks of yeast. Nature Genetics 24, 355–361.

[38] Dayton, E., Konings, D., Powell, D., Shapiro, B., Butini, L., Maizel, J. and Dayton, A. (1992) Extensive sequence-specific information throughout the CAR RRE, the target sequence of the human-immunodeficiency-virus type-1 rev protein. Journal of Virology 66, 1139–1151.

[39] Baudin, F., Marquet, R., Isel, C., Darlix, J., Ehresmann, B. and Ehresmann, C. (1993) Functional sites in the 5′ region of human-immunodeficiency-virus type-1 RNA form defined structural domains. Journal of Molecular Biology 229, 382–397.

[40] Powell, D., Zhang, M., Konings, D., Wingfield, P., Stahl, S., Dayton, E. and Dayton, A. (1995) Sequence specificity in the higher-order interaction of the rev protein of HIV with its target sequence, the RRE. Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology 10, 317–323.

[41] Jackson, R. and Kaminski, A. (1995) Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. RNA 1, 985–1000.

[42] Mandl, C., Holzmann, H., Meixner, T., Rauscher, S., Stadler, P., Allison, S. and Heinz, F. (1998) Spontaneous and engineered deletions in the 3′ noncoding region of tick-borne encephalitis virus: construction of highly attenuated mutants of a flavivirus. Journal of Virology 72, 2132–2140.

[43] Okamura-Ikeda, K., Fujiwara, K. and Motokawa, Y. (1992) Molecular-cloning of a cDNA-encoding chicken T-protein of the glycine cleavage system and expression of the functional protein in *Escherichia coli* – effect of messenger RNA secondary structure in the translational initiation region on expression. Journal of Biological Chemistry 267, 18284–18290.

[44] Pati, S., Disilvestre, D. and Brusilow, W. (1992) Regulation of the *Escherichia coli* unch gene by messenger RNA secondary structure and translational coupling. Molecular Microbiology 6, 3559–3566.

[45] Manzella, J.R.W., Ryehlik, W., Rhoads, R., Hershey, J. and Blackshear, P. (1991) Insulin induction of ornithine decarboxylase – importance of messenger RNA secondary structure and phosphorylation of eukaryotic initiation factor EIF-4b and factor EIF-4e. Journal of Biological Chemistry 266, 2383–2389.

[46] Vandeguchte, M.V.T., Kok, J. and Venema, G. (1991) A possible contribution of messenger-RNA secondary structure to translation initiation efficiency in *Lactococcus lactis*. FEMS Microbiology letters 81, 201–208.

[47] Fontana, W. (2002) Modelling 'evo-devo' with RNA. Bioessays 24, 1164–1177.

[48] Rutherford, S. and Lindquist, S. (1998) Hsp90 as a capacitor for morphological evolution. Nature 396, 336–342.

[49] Summers, K. (1996) Relationship between genotype and phenotype in monogenic diseases: relevance to polygenic diseases. Human Mutation 7, 283–293.

[50] Weatherall, D.J. (2001) Phenotype–genotype relationships in monogenic disease: lessons from the thalassaemias. Nature Reviews Genetics 2, 245–255.

[51] Lauerer, M., et al. (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with antisense rbcS. 6. Effect on photosynthesis in plants grown at different irradiance. Planta 190, 332–345.

[52] Mayr, E. (1982) The Growth of Biological Thought, Belknap Press, Cambridge, MA.

[53] Schultes, E. and Bartel, D. (2000) One sequence, two ribozymes: Implications for the emergence of new ribozyme folds. Science 289, 448–452.

[54] Aharoni, A., Gaidukov, G., Khersonsky, O., McQ Gould, S., Roodveldt, C. and Tawfik, D. (2005) The 'evolvability' of promiscuous protein functions. Nature Genetics 37, 73–76.

[55] Tomarev, S. and Piatigorsky, J. (1996) Lens crystallins of invertebrates – diversity and recruitment from detoxification enzymes and novel proteins. European Journal of Biochemistry 235, 449–465.

[56] Piatigorsky, J. (1998) Gene sharing in lens and cornea: facts and implications. Progress in Retinal and Eye Research 17, 145–174.

[57] Stone, J. and Wray, G. (2001) Rapid evolution of *cis*-regulatory sequences via local point mutations. Molecular Biology and Evolution 18, 1764–1770.

[58] Clark, A.G., et al. (2003) Inferring nonneutral evolution from human–chimp–mouse orthologous gene trios. Science 302, 1960–1963.

[59] King, M.C. and Wilson, A.C. (1975) Evolution at two levels in humans and chimpanzees. Science 188, 107–116.

[60] A. Wagner, Princeton University Press, Princeton, NJ, 2005.