OPINION

Neutralism and selectionism: a network-based reconciliation

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Abstract | Neutralism and selectionism are extremes of an explanatory spectrum for understanding patterns of molecular evolution and the emergence of evolutionary innovation. Although recent genome-scale data from protein-coding genes argue against neutralism, molecular engineering and protein evolution data argue that neutral mutations and mutational robustness are important for evolutionary innovation. Here I propose a reconciliation in which neutral mutations prepare the ground for later evolutionary adaptation. Key to this perspective is an explicit understanding of molecular phenotypes that has only become accessible in recent years.

The tension between neutralism and selectionism is at least as old as the field of molecular evolution[1]. Most of the historical debate between neutralism and selectionism was centred on explanations for genetic variation in populations. In this context, neutralists and selectionists agreed that deleterious mutations occur frequently in evolving molecules, but they profoundly disagreed on the relative importance of effectively neutral and beneficial mutations. To neutralism, beneficial mutations are rare and are fixed less frequently than neutral or slightly deleterious mutations[2]. By contrast, according to selectionism, beneficial mutations are abundant: most mutations that go to fixation in a population would be beneficial, or are at least linked to abundantly occurring beneficial mutations. Selectionists such as Ernst Mayr dismiss the importance of neutral evolutionary change altogether[3] and some have pronounced neutralism dead[4]. By contrast, prominent voices persist in their support of it[5,6]; recent positions emphasize the importance of demographic factors such as population size[7] (BOX 1).

Although the neutralism–selectionism tension about genetic variation has abated, the underlying tension persists. It has implications that go far beyond explanations of genetic variation. One of these regards the origin of evolutionary innovations, one of the most fundamental unsolved problems of evolutionary biology. Innovations consist of molecular phenotypes with novel functions and structures, including new protein and RNA structures, new gene expression states of regulatory gene networks or new metabolic capabilities of metabolic networks. According to the selectionist perspective, such innovations might arise through beneficial mutations, which change the properties of a molecule or network when they first arise and that constitute the innovation. According to the neutralist perspective, innovations might be facilitated by mutations that do not affect the functions of a molecule when they first arise.

> “…both the neutralist emphasis on neutral mutations and the selectionist emphasis on beneficial mutations capture equally important aspects of biological reality.”

The discussion below makes a case for how to resolve the neutralism–selectionism tension, and also suggests that this tension has persisted for a reason: to resolve it requires a detailed understanding of molecular phenotypes, such as protein structures, that because of their complexity are poorly captured by the scalar representation of phenotypes and fitness that is characteristic of population genetic models. Analysis of such complex molecular phenotypes has become possible only recently.

The mounting case against neutralism

Even before genome-scale evidence became available, data based on individual genes and their evolution argued against neutralism. Such data came from deviations of the constant rate of molecular evolution predicted by the neutral theory[8], as well as from patterns of nucleotide variation within and among populations[4,9,10].

Genome-scale data from protein-coding regions and even from some non-coding DNA has strengthened the case against neutralism. For instance, the McDonald–Kreitman test[8] provides evidence that between 30 and more than 90 percent of nucleotide changes in the *Drosophila* genus and in other organisms go to fixation because they are beneficial[11–21].

A second line of evidence comes from the relationship between the mean number of polymorphic differences between alleles within a species, π, and the number of fixed differences between genes in two species, d. For neutral mutations, a positive association between π and d should exist, because the neutral theory predicts that both quantities are linearly proportional to the rate at which neutral mutations arise. Recent genome-scale data shows instead that this association is in fact negative[17,22]. Selectionism can readily explain this association[22]: alleles that are genetically linked to a beneficial mutation that sweeps through a population will ‘hitchhike’ to fixation with this mutation, because recombination cannot decouple them rapidly enough from this mutation during a selective sweep[23,24]. Thus, genomic regions in which such selective sweeps are frequent should show decreased polymorphisms

Box 1 | **Genetic drift and the importance of population size**

The neutral theory of molecular evolution and its offshoots aim to predict the fate of neutral mutations in populations[1]. This fate is influenced by genetic drift, a force of evolutionary change that is strongest in small populations. In any finite population, the frequency (p) of an allele fluctuates from generation to generation, because alleles get sampled from the previous generation to form the next. Such sampling fluctuations are strongest in small populations, in which the number of alleles to be sampled is small. For example, in diploid organisms the variance in the amount of change in allele frequency from generation to generation is given by $V = p(1 - p)/2N$ (REF. 95), where N is the effective population size. The expression for V above shows that allele-frequency fluctuations are larger in small populations.

Genetic drift affects several population genetic processes. For example, a newly arisen neutral allele that eventually goes to fixation takes on average $4N$ generations to do so in a diploid population[95]. The fate of mutations with a selection coefficient that is much smaller than $1/(4N)$ is determined by drift rather than by selection, because the generation-to-generation random allele-frequency fluctuations are stronger than the influence of selection. Even mutations with a selection coefficient that is greater than $1/(4N)$ can be influenced by drift: weakly deleterious mutations can go to fixation, whereas weakly beneficial mutations can be lost. If a neutral mutation occurs physically close to a beneficial mutation, then the neutral mutation might be rapidly swept to a high frequency or to fixation, if its association with the beneficial mutation is not broken up by recombination. The effects of such 'hitchhiking'[24] or 'genetic draft'[9] amount to a reduction of N experienced by alleles that occur in a region where such selective sweeps are frequent[9]. Alternatively, if a neutral mutation occurs close to a region where deleterious mutations segregate, the neutral mutation might be dragged to extinction along with the deleterious mutations. This phenomenon of 'background selection'[96] can affect polymorphisms and time to fixation of neutral alleles. Because recombination rates vary substantially among organisms and chromosomal regions[7,97], the impact of these phenomena on allelic variation might also vary.

Effective population sizes vary by some five orders of magnitude, from values of 10^4 for vertebrates to values of up to 10^9 for prokaryotes; they are generally lower in larger, multicellular organisms[7]. Many alleles that are subject to selective forces in prokaryotes might be evolving neutrally by genetic drift in vertebrates. The consequences may be far-reaching. For example, Lynch recently argued that the emergence of the complex genome architecture of higher organisms, including the rising abundance of introns and transposable elements, might have centrally involved the relaxation of selection caused by their smaller population sizes[7].

(low π). By contrast, abundant adaptive mutations should increase the allelic divergence d in a genomic region, because a rapid succession of allele substitutions driven by beneficial mutations will occur in such regions. The net result is that π and d should be negatively related, as in genome-scale data and in contrast to the neutralist view.

Other patterns of molecular evolution that are more easily explained by a selectionist perspective include larger amounts of nucleotide polymorphisms in genomic regions with higher recombination rates, and the absence of a strong correlation between intrapopulation genetic diversity and population size (the 'paradox of variation'). Some of these patterns are reviewed in REF. 22.

Genome-scale sequence data is the gold standard of comparative data, the most comprehensive kind of data that can inform the neutralism–selectionism debate. That it strongly argues in favour of abundant beneficial mutations does not bode well for neutralism. However, in contrast to work focused on genotypes, analyses of molecular phenotypes indicate that neutral change might be important for evolutionary innovation. Below I will discuss these recent developments, and suggest a synthetic perspective on both classes of evidence. Essential to this perspective is that neutral mutations are key to prepare the ground for later evolutionary adaptation. From this perspective, both the neutralist emphasis on neutral mutations and the selectionist emphasis on beneficial mutations capture equally important aspects of biological reality.

How neutrality facilitates innovation

Laboratory studies. I will frame the discussion by using the concept of genotype networks, or neutral networks (BOX 2) — these are connected sets of genotypes that share the same phenotype. Because neutral networks are abstractions they come with several limitations, but they are immensely useful to sharpen our intuition about molecular evolution. They have already helped to explain observations as different as the evolution of viral antigens and new ribozyme functions[25–27].

The human hepatitis delta virus encodes a ribozyme that carries out a self-cleavage reaction that is necessary to complete the viral life cycle. This ribozyme is unrelated in sequence, structure and enzymatic activity from the class III self-ligating ribozyme, which is a synthetic ligase isolated from a pool of random RNA molecules[25]. Schultes and Bartel succeeded in transforming these markedly different molecules into one another through a mutational walk through sequence space that required some 40 mutations[25]. Importantly, through most of this walk the enzymatic activity of the mutated molecule does not change dramatically, providing evidence for a genotype network for enzyme activity. Halfway through this walk, four mutations alone were sufficient to change the activity of one enzyme into that of the other. These observations suggest that the invariance of a phenotype in the face of many mutations facilitates the evolution of new ribozyme functions.

Several recent laboratory studies involving mutations in multiple enzymes highlight the importance of neutral change from a different angle[28–31]. One study[28] examined the evolution of new functions of the enzyme cytochrome P450. Error-prone PCR was used to introduce multiple mutations into different enzyme variants, which differed in thermodynamic stability and in robustness to mutations. The more robust a molecule is, the more likely it is that mutations in it are neutral, and that they do not change the structure and function of the molecule[28,32]. Strikingly, the stable and more robust variants of cytochrome P450 more readily evolved the ability to hydrolyse new substrates.

A different line of evidence comes from laboratory evolution studies of the serum protein paraoxonase[29,31]. This enzyme is primarily a lactonase, but can also catalyse reactions involving other substrates. Error-prone PCR mutagenesis was used in an effort to increase the activity of these side reactions. Surprisingly, many mutations that increase these side activities dramatically (10^1–10^6-fold) are neutral with respect to the primary activity. Similar observations were made for other enzymes, such as a bacterial phosphotriesterase and carbonic anhydrase II[29].

In addition, some 300 paraoxonase variants that are neutral or close to neutral with respect to the primary activity of the enzyme were mutationally closer to new phenotypes such as thiolactonase and phosphotriesterase[31]. Thus, for this protein also, neutral sequence changes facilitate evolutionary adaptation.

Evolutionary studies. The studies described above explored the evolution of new molecular functions on short laboratory timescales. These conclusions can be extrapolated to larger, evolutionary timescales: more robust molecules have evolved a greater diversity of functions. A recent study on the functional diversity of protein domains took advantage of the ability to estimate the robustness of a protein structure to mutations, either from its distribution of amino-acid contacts[33], or through the amount of amino-acid variation observed within the protein[34]. Robustness is highly positively associated with the functional diversity of proteins, either as estimated through the diversity of enzymatic reactions catalysed by enzyme families[34] (FIG. 1), or through more general indicators of diversity, such as gene ontology functional annotations[34,35].

Protein scaffolds. Thus, on both short and long timescales, the ability of a molecule to undergo neutral change facilitates the evolution of new functions in it. Observations from such systematic studies are supplemented by more anecdotal evidence. One such line of evidence comes from protein engineering, in which mutagenesis creates proteins with new functions from existing protein scaffolds. Desirable in this process are scaffolds with a structural backbone that is insensitive to mutations, and that can thus be modified through the substitution of many different amino acids. One of the most successful scaffolds of this type is the zinc-finger domain[36], which is strikingly robust to mutations. When all but seven of its 26 amino acids are replaced by alanine, the structure is left essentially intact[37]. This robustness accounts for the great versatility of this domain in protein engineering, in which it can be used to design proteins with a great variety of DNA-binding activities and molecular functions[38]. It is perhaps no coincidence that the zinc-finger domain is also the most abundant domain in the human proteome — 4,500 zinc-finger domains are found in more than 500 proteins[39].

Gene duplications. Further anecdotal evidence comes from the role of gene duplications in morphological evolution. Gene duplications increase the incidence of neutral mutations in the duplicated genes. For example, shortly after a duplication, the ratio of amino-acid replacement to silent nucleotide changes is greatly elevated in duplicate genes[40,41]. Gene and genome duplications are associated with striking evolutionary innovations in the history of life, such as the diversification of the vertebrate body plan, the radiation of flowering plants and the evolution of highly integrated organs, such as the four-chambered mammalian heart[42–45]. This association, although circumstantial, is fully consistent with the notion that the increased potential for neutral change caused by gene duplication facilitates evolutionary adaptation[46].

Regulatory networks. Some of the principles gleaned about the relationship between neutrality and the ability to evolve also apply to systems on levels of biological organization higher than the molecular level. For example, computational work has shown that transcriptional regulation circuits might form extended neutral networks in which the phenotype — a gene expression pattern — might be preserved despite extensive genetic change in regulatory interactions[47]. A recent experimental study rewired the *Escherichia coli* transcriptional regulation network[48] in almost 600 different ways by introducing novel regulatory interactions. Most of the rewired networks showed no growth difference to the ancestral network in several different environments.

Such tolerance of networks to regulatory change has also been implicated in the evolution of yeast mating-type control. Yeast cells show two different sexes called the a and α mating types, which are distinguished by the expression of mating type-specific genes. Distantly related yeasts regulate the expression of these genes in

Box 2 | **Neutral networks**

Consider a space of discrete macromolecular sequences (genotypes) of *n* monomers, such as RNA or proteins. In this space, two sequences are 'immediate neighbours' if they can be transformed into one another by a single point mutation that changes only one monomer. A genotype network, or neutral network[98], is a set of genotypes that share a common phenotype. The phenotype could be a molecular structure (RNA or protein structure) or function (such as enzymatic activity). In addition, each pair of genotypes in the network can be connected through a series of single point mutations that do not leave the network. The exploration of such networks became possible only with the ability to characterize molecular phenotypes for many different genotypes, either computationally or experimentally. The germ of this concept originated with Maynard-Smith[99] and was developed by Schuster and collaborators[98], who showed that the neutral networks associated with many individual RNA secondary structures are vast and span sequence space. The concept was later applied to proteins[100–103], and to higher-order molecular systems, such as regulatory networks[47].

Robustness and network size

Because even genotypes of moderate length *n* can have a huge number of phenotypes (for example, ~1.8^n RNA secondary structures)[104,105], a sequence space is filled by a myriad of neutral networks, each one associated with a different phenotype. These phenotypes have very different neutral network sizes. Some phenotypes are adopted by many sequences. The neutral network of such phenotypes is large, and the average sequence on such a large network has many immediate neighbours. Other phenotypes are adopted by fewer sequences. Their neutral networks are smaller, and sequences on them have fewer immediate neighbours[50,106]. So the larger the phenotype's neutral network, the greater the phenotype's robustness to mutations that change single amino acids or nucleotides. The existence of neutral networks has many implications for the evolutionary dynamics of populations of sequences[26,27,54,107].

Population size and neutral networks

Population sizes play an important part in the evolutionary dynamics associated with neutral networks. For any given molecular system the neutral network associated with a given phenotype will be larger in small populations, because selection is less effective (BOX 1). Conversely, in very large populations, a huge number of mutations occur per generation. This means not only that populations diffuse more rapidly on any given network, but that they may also be able to vault any fitness 'valley' that separates two neutral networks by virtue of double or multiple mutations that co-occur in single individuals[62,90]. There are many open questions about how population size, neutral network size, mutation rates and recombination rates interact and affect evolutionary dynamics.

markedly different ways. For example, in the yeast *Candida albicans*, a-specific genes are expressed in a-cells by a transcriptional activator. Their unexpressed state in α-cells is the default state. By contrast, in the yeast *Saccharomyces cerevisiae*, the a-specific genes are expressed by default. They must be transcriptionally repressed in α-cells. A recent study[49] showed how a series of genetic changes in transcription factors and regulatory regions can change this regulatory mechanism without changing the regulatory phenotype. Taken together, observations such as these suggest that the kind of neutrality that aids the evolution of new functions in molecules also occurs in regulatory gene networks.

A population perspective. All evolutionary processes occur in populations. A population perspective might thus be helpful to understand how neutrality may facilitate evolutionary innovation, as the above observations suggest. Consider a hypothetical population of molecules on a phenotype's neutral network (BOX 2). The 'genotypic neighbourhood' of this population is the set of molecules that are just one nucleotide or amino-acid change away from any population member. This neighbourhood contains numerous different genotypes, many of which have phenotypes that are different from those of the population's members. These phenotypes comprise the range of phenotypic variation that is readily accessible to the population, via only one mutational change. Only a small fraction of these phenotypic variants may be beneficial. Populations with many different phenotypes in their genotype neighbourhood may, through blind mutational change, 'discover' more readily one of these beneficial variants than populations with few different phenotypes in their neighbourhood.

If a population that is initially genetically homogeneous evolves through cycles of mutation and selection, it will spread out through the neutral network and become more genetically diverse. Once it has spread out, the diversity of phenotypes in its neighbourhood will also increase, until it reaches a steady state. How does this diversity depend on the average fraction of mutations in network sequences that are neutral? This is a key question linking the propensity to produce novel phenotypes with neutrality.

This question has recently been addressed in a computational study using RNA secondary structures as phenotypes[50]. This study compared evolving populations with phenotypes of different robustness, that is, different neutral network size and different average fraction of neutral mutations per population member (BOX 2). Populations with members that have a robust RNA phenotype are genetically more diverse, and also show much greater phenotypic diversity in their genotypic neighbourhood, than populations with a less robust phenotype. This result can be explained as follows (FIG. 2): individual sequences on a large neutral network have, on average, more neutral neighbours than sequences on a small neutral network. They will thus experience fewer deleterious mutations, which would cause them to be eliminated from the population. With fewer deaths, the population remains more diverse, and spreads more rapidly through the neutral network. Its genotypic neighbourhood contains a richer spectrum of different phenotypes. Although populations of small size or low mutation rates are genetically homogeneous most of the time, this principle holds in modified form also for such populations[50]. The model crucially depends on one thing: the neighbourhood of different genotypes on a neutral network must contain different phenotypes. This is an observation that holds not only for molecular phenotypes[51,52], but also for regulatory systems[53]. The core suggestion of FIG. 2 — that neutrality facilitates evolutionary innovation and adaptation — agrees with the observation that robust molecules tolerating many neutral mutations more readily evolve new functions both on laboratory and evolutionary timescales.

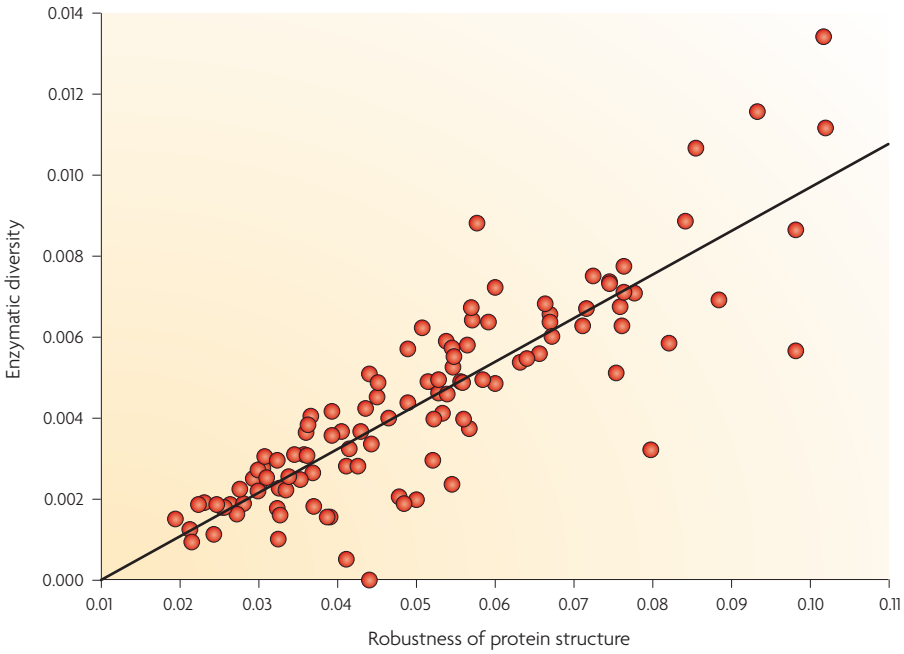

Figure 1 | **Protein structures that are highly robust to mutations evolve greater functional enzymatic diversity.** The horizontal axis shows the length-normalized largest eigenvalue of the contact density matrix for 112 ancient protein domains[33], which is a measure of the number of sequences adopting the protein's structure, and thus also is a measure of the protein's robustness to mutations. The vertical axis shows a measure of the number of enzyme families associated with a protein fold, known as the enzymatic diversity (*D*). Enzyme families are distinguished through the substrates they use, and through structural similarities in their active sites[108]. If a set of proteins with the same fold has a number of different enzymatic functions (*i*), and if p_i is the frequency with which each function occurs in this set, then $D = -\Sigma_i p_i \log p_i$. The data show that robust domains have evolved greater functional diversity. The same is observed for other indicators of robustness and diversity[34,35]. The graph was produced using data from REF. 34.

Reconciling neutralism and selectionism

Selectionism is supported by patterns of genomic evolution. The importance of robustness and neutral change is highlighted by studies of molecular phenotypes. FIGURE 3 suggests how to reconcile these observations in the form of a conceptual model centred on the neutral network metaphor. Consider first, for simplicity, a single genotype (sequence

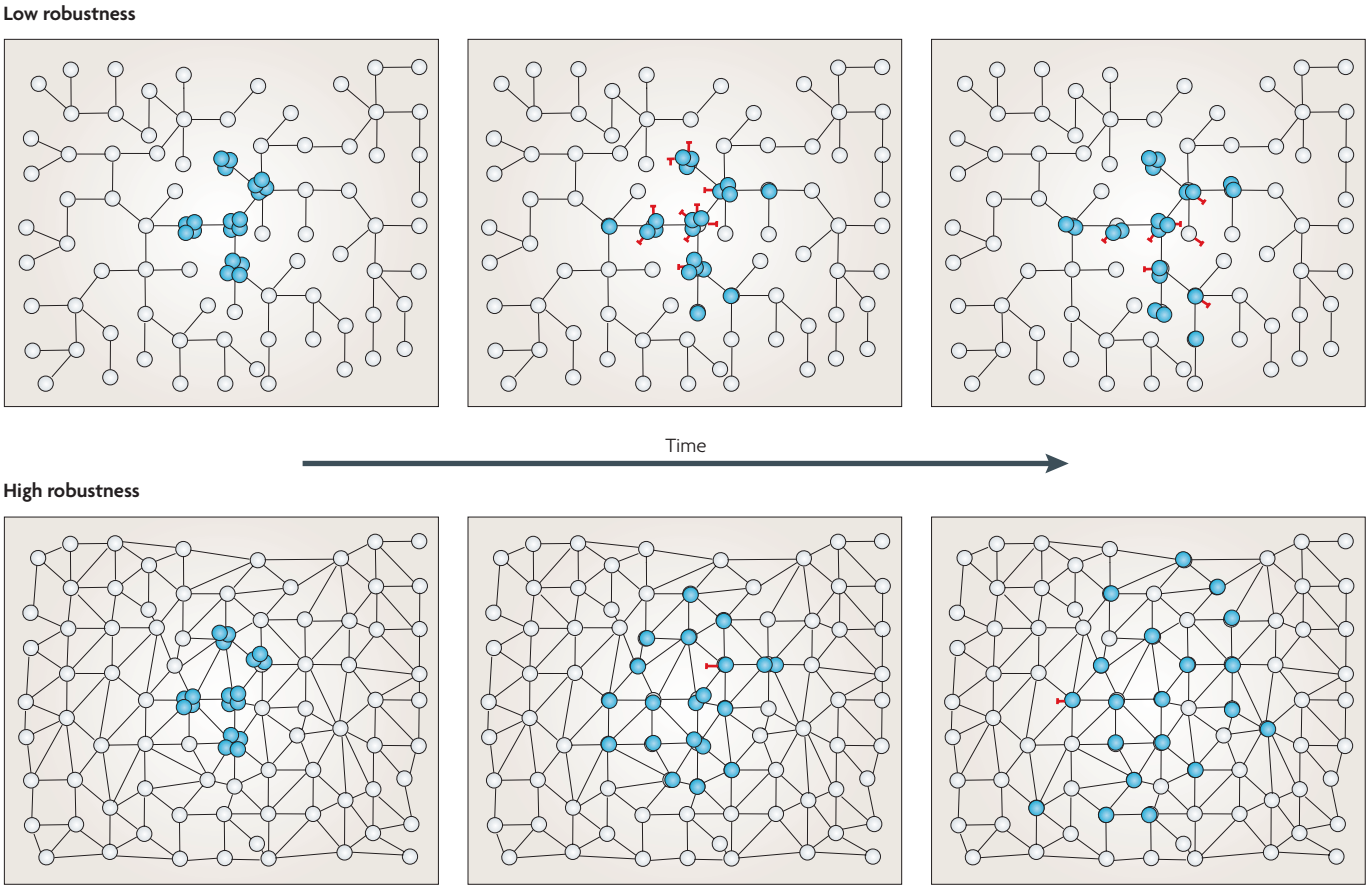

Figure 2 | **Robust phenotypes can lead to a rapid yet neutral exploration of sequence space.** Each rectangle represents part of a space of genotypes. Grey circles correspond to individual genotypes on a neutral network. A straight line links two genotypes if they can be interconverted through a single point mutation. The top three panels show how neutral evolution explores genotype space for a phenotype with low robustness. This is a phenotype with a small neutral network, in which individual genotypes have, on average, few neutral neighbours. The bottom three panels show the same evolutionary process, but for a robust phenotype. All panels contain the same number of genotypes to facilitate comparison. They therefore do not reflect neutral network sizes, but merely the fact that genotypes on large neutral networks also tend to have more neutral neighbours. Blue circles correspond to individual members of a population, and red stubs illustrate hypothetical deleterious mutations that cause a mutation to genotypes lying outside the neutral network (not shown). The differences in the number of red stubs between the top and bottom panels illustrates that robust phenotypes with large neutral networks are subject to fewer deleterious mutations. The two leftmost panels show two genetically identical populations with moderate genotypic diversity, in which several individuals have the same genotype. As these populations evolve through mutation and selection confining them to a network, the population with the robust phenotype will spread more rapidly, because fewer deleterious mutations impede accumulation of genotypic diversity. This phenomenon is independent of population size or mutation rate[109].

or otherwise) that undergoes random mutational change, a random walk on the neutral network of the phenotype it adopts. Assume that this phenotype is suboptimal, and that the target of the evolutionary search is a better, optimal phenotype. The sequence might first take several neutral mutational steps on the neutral network (I will focus on neutral change, although deleterious change may actually be more likely). After some steps, a phenotype-changing beneficial mutation might produce a new phenotype closer to the target phenotype. The random walker will thus hop from the first to a second neutral network. From then on, the cycle repeats. A number of neutral mutations — an exploration of a current neutral network — would be followed by a mutation that 'discovers' a new phenotype closer to the target.

The overall scenario is similar for evolving populations instead of evolving single genotypes. The population explores one neutral network until one of its members uncovers a phenotype and neutral network closer to the target, through a beneficial mutation that then sweeps to fixation in the population. Previously occurred mutations that paved the way for this sweep would 'hitchhike' to fixation[9,24]. After this sweep, the descendants of this successful mutant explore the neutral network until one of them finds a new and better phenotype, and so on. Thus, evolutionary adaptation proceeds by cycles of exploration of a neutral network (a neutralist regime), and dramatic diversity reduction as beneficial mutations discover new phenotypes residing on new neutral networks (a selectionist regime). In this context, strong selectionism would demand that no neutral mutations would occur between phenotypic changes. Every single change would be either deleterious (and hence eliminated) or it would discover a new genotype network closer to the target phenotype.

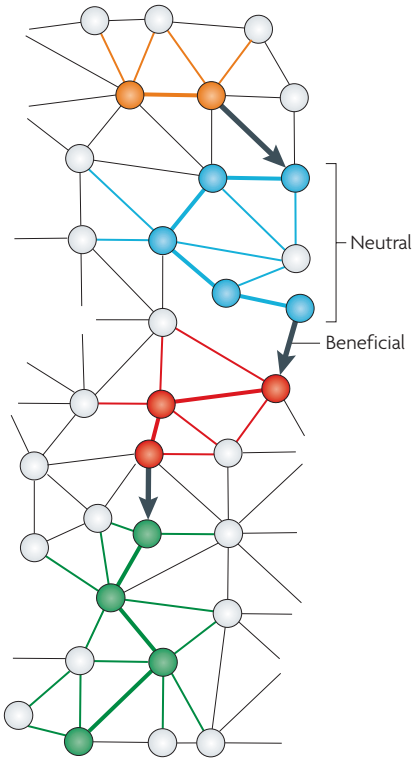

Figure 3 | **Cycles of neutral evolution and positive selection through traversal of multiple networks in adaptive evolution.** Grey circles correspond to individual genotypes. A straight line links two genotypes if they can be interconverted through a single point mutation. For simplicity, the path (thick edges) of only a single genotype through sequence space is shown. The genotype evolves towards a hypothetical adaptive phenotype (not shown). In order to arrive at this phenotype, it traverses four different neutral networks (coloured nodes; thin coloured edges point to neighbours on the same network that are bypassed by the genotype). Within each neutral network, evolution is neutral, but at the transition between neutral networks (indicated by the arrows) positive selection occurs.

Let us now focus on the last mutation in a sequence of neutral steps, and on the beneficial mutations that follow it. Importantly, the phenotypic effect of this beneficial mutation might depend on the mutation(s) preceding it. FIGURE 4 shows a hypothetical example involving RNA structure phenotypes, in which a first mutation (C to U at position 30) in a sequence leaves the minimum free-energy structure of an RNA molecule unaffected. A second mutation (C to U at position 39) then changes this secondary structure. The first mutation is neutral, but the second mutation is non-neutral, for example, it might be beneficial. What if these two mutations had occurred in the reverse order (position 39 followed by 30). The C to U mutation at position 39 is, by itself, neutral (FIG. 4). However, if the C to U mutation at position 30 follows this change, the same phenotypic change results as with the previous mutation order. In other words, if the sequence of mutations had been reversed, we would now call the mutation at position 39 neutral, as opposed to beneficial. Similarly, the mutation at position 30 would now be beneficial, although it was previously neutral. The situation would be even more complicated if we considered additional mutations that the molecule experienced earlier. Similar observations also hold for neutral and deleterious mutations, or for deleterious and beneficial mutations. The effect of a mutation exists only in the context of the mutations preceding it.

If the effect of a mutation depends on the evolutionary history of the genotype, it could be argued that that it is not sensible to speak of neutral, deleterious or beneficial mutations at all. However, this would be an overreaction. The notion of a deleterious mutation is clearly necessary in characterizing the causes of genetic diseases, as is the notion of a beneficial mutation to characterize evolutionary adaptations. However, we need to acknowledge a key limitation of these notions. A mutation has an effect at the time at which it arises, and this effect may change over time: a mutational change that was once neutral might later become beneficial (or deleterious) depending on other genetic changes. This view stands in stark contrast to how many models of population genetics — the quantitative theory that aims to explain biological evolution — represent genetic change. There, with important exceptions (see REF. 9 for an example), individual alleles are often labelled as unchangingly deleterious, neutral or beneficial.

Boom and bust cycles of diversity. If the conceptual model from FIG. 3 captures the evolutionary dynamics of biological systems, then three classes of predictions follow. The first is that evolutionary change should often occur in cycles of neutral diversity expansion and selective diversity contraction. RNA molecules evolving towards a target structure demonstrate this kind of dynamics in computational work[54]. This type of dynamics has also been observed in sufficiently well sampled evolving populations in the wild. For example, haemagglutinin, a key viral antigen of the human influenza virus, shows punctuated and episodic evolution in its antigenic properties. Episodes of small genetic changes with large effect on the antigenic phenotype alternate with periods in which genetic variation accumulates with little phenotypic change. A neutral network model can best explain these features of haemagglutinin evolution[26,27]. More generally, the closer an evolving population gets to an optimal molecular phenotype the longer it might take to find further phenotypic improvements[54], because the diversity expansion phase of a cycle will also increase. Anecdotal evidence is available from laboratory evolution studies that indicates that this holds not only for molecular phenotypes but also for more complex phenotypes, such as cell size[55]. Observations such as these argue against a strong selectionist perspective, in which no neutral mutations and no phenotypic stasis should occur between phenotypic changes.

Pervasive epistasis. A second class of prediction is that consecutive mutations in molecules should show interdependent effects. The relationship between the two mutations in FIG. 4 is a special case of epistasis[56,57]. Most genetic analyses of epistasis have focused on mutations that are induced experimentally or that are already present in a population. Until recently[58–65], the temporal aspect of epistasis highlighted above, in which past mutations influence the effects of future mutations, has received less attention. Several recent studies observe that mutational effects frequently change over time. For instance, a recent computational study demonstrated that the effects of mutations on the kind of molecular structure shown in FIG. 4 can influence the evolutionary dynamics of RNA molecules[66]. In this study the authors introduced mutations with small deleterious effects on an RNA-structure phenotype into an RNA molecule, and recorded the evolutionary trajectories of these changes in a finite population. The incidence of fixation was significantly higher than predicted by theory[1]; this is partly due to compensatory mutations[66,67]. Such compensatory mutations are also obvious from phylogenetic analyses of RNA and protein evolution. For instance, as many as 50% of deleterious mutations in human transfer RNA genes occur in normal transfer RNAs of other mammals[59]. Such epistasis is also called sign epistasis, because the fitness effects of successive mutations have different signs (positive, neutral or negative)[61,68]. Sign epistasis is frequent in

fruitflies[58] and humans, in which 10% of disease-causing mutations are found as wild-type variants in other mammals[60]. Mechanistic explanations for its pervasiveness have been proposed[69–71].

Detailed functional analyses can demonstrate the epistatic interactions of specific mutations. A case in point is the evolution of the mineralocorticoid receptor (which is activated by the hormone aldosterone), which originated via a gene duplication from the glucocorticoid receptor (which is activated by cortisol). Recent studies identified the mutations responsible for the cortisol specificity of the glucocorticoid receptor[72,73]. This was done by reconstructing the common ancestor of both receptors, which responds to both aldosterone and cortisol, and identifying mutations that confer reduced sensitivity to aldosterone but retained sensitivity to cortisol. Two mutations stood out: serine at position 106 replaced by proline (S106P) and leucine at position 111 replaced by glutamine (L111Q). L111Q followed by S106P yields a receptor that is still sensitive to cortisol but that is 1,000-fold less sensitive to aldosterone[72,74]. This mutant combination is another example of the epistatic interactions highlighted above. L111Q has little effect by itself, but in combination with the mutation S106P it facilitates the evolution of a molecular specialization that has served tetrapods well for many millions of years. Several other mutation pairs have this property[72,73].

Studies such as these can also help to elucidate the mechanistic reasons why mutations with weak effects can acquire strong effects in combination with other mutants. For example, the above L111Q change introduces a new amino-acid side chain. However, this new side chain matters only after the S106P change, which repositions this new side chain. The result is that the side chain at position 111 can now form a hydrogen bond with a hydroxyl group of cortisol, which aldosterone lacks. The result is a cortisol-specific interaction between protein and ligand. Another general theme, which has been best explored for enzymes, is that mutations that introduce a novel enzymatic activity often also destabilize the structure of the protein. Thus, mutations that precede or follow these function-changing mutations, and that stabilize the enzyme (but themselves do not introduce a novel activity) can be important for functional innovation[69–71,75].

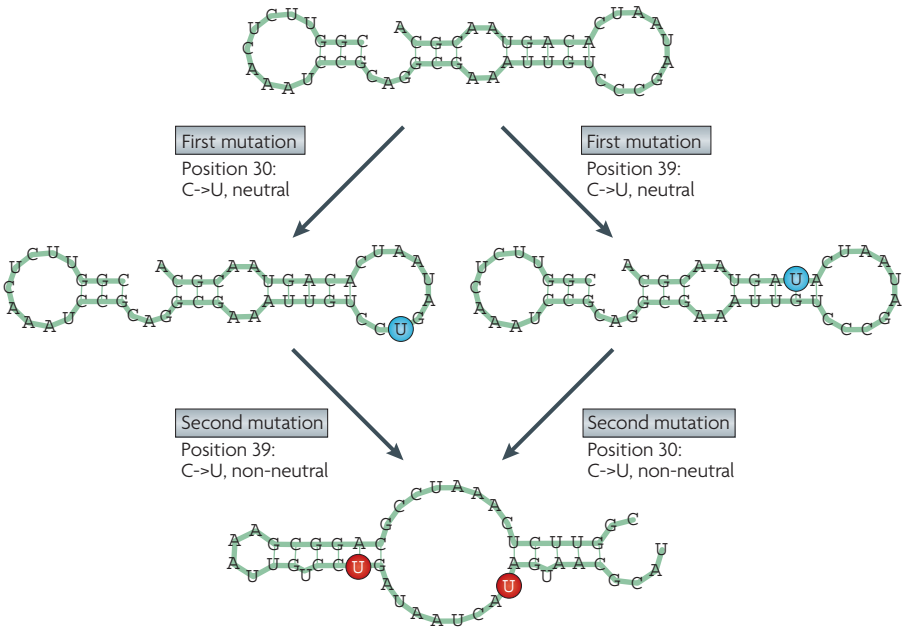

Figure 4 | **Neutrality of mutations depends on the order in which the mutations occur.** The figure focuses on two mutations in a sequence, shown together with its minimum free-energy secondary structure[110]. They are C to U transitions at positions 30 (mutation 1) and 39 (mutation 2). By itself, mutation 1 is neutral, as is mutation 2. However, when mutation 1 is followed by mutation 2, or vice versa, a changed secondary structure results.

Glossary

Effective population size
Indicates how many individuals actually contribute alleles to the next generation, as opposed to the actual number of individuals in a population. For various reasons, including the preferential reproduction of some individuals and population size fluctuations over time, the effective population size is typically smaller than the actual number of individuals in the population.

Eigenvalue
For a matrix A and a vector v, an eigenvalue c is a scalar that obeys the equation Av = cv.

Epistasis
The dependency of the effects of a mutation on mutations in other parts of a gene or genome.

Gene ontology
A widely used classification system of gene functions and other gene attributes that uses a controlled vocabulary.

Maximum-likelihood estimation
A statistical method for fitting mathematical models to data. It is widely used to estimate the structure of phylogenetic trees from sequence data.

McDonald–Kreitman test
A statistical test that can detect positive selection based on intra- and interpopulation divergence of nucleotide changes in proteins.

Molecular phenotype
A phenotype is any observable trait or feature of an organism other than the DNA itself (that is, the genotype). Molecular features, such as the structure of a particular proteins, are molecular phenotypes.

Mutational walk
A series of small mutational changes in sequence space.

Positive selection
Also known as directional selection. A process by which natural selection favours a single beneficial genotype over other genotypes and may drive this genotype to a high frequency in a population.

Selection coefficient
The fitness difference of a genotype compared with the wild-type genotype.

Selective sweep
When a mutation with beneficial fitness effects arises in a population, natural selection may drive or sweep this mutation to a high frequency or to fixation (a frequency of 100%) within a short amount of time.

Sequence space
All DNA, RNA or amino-acid sequences of a given length, that is, a given number of monomers.

Zinc-finger domain
A protein domain in which a zinc ion is bound to two conserved cysteine and histidine residues, an interaction that stabilizes the structure of the domain.

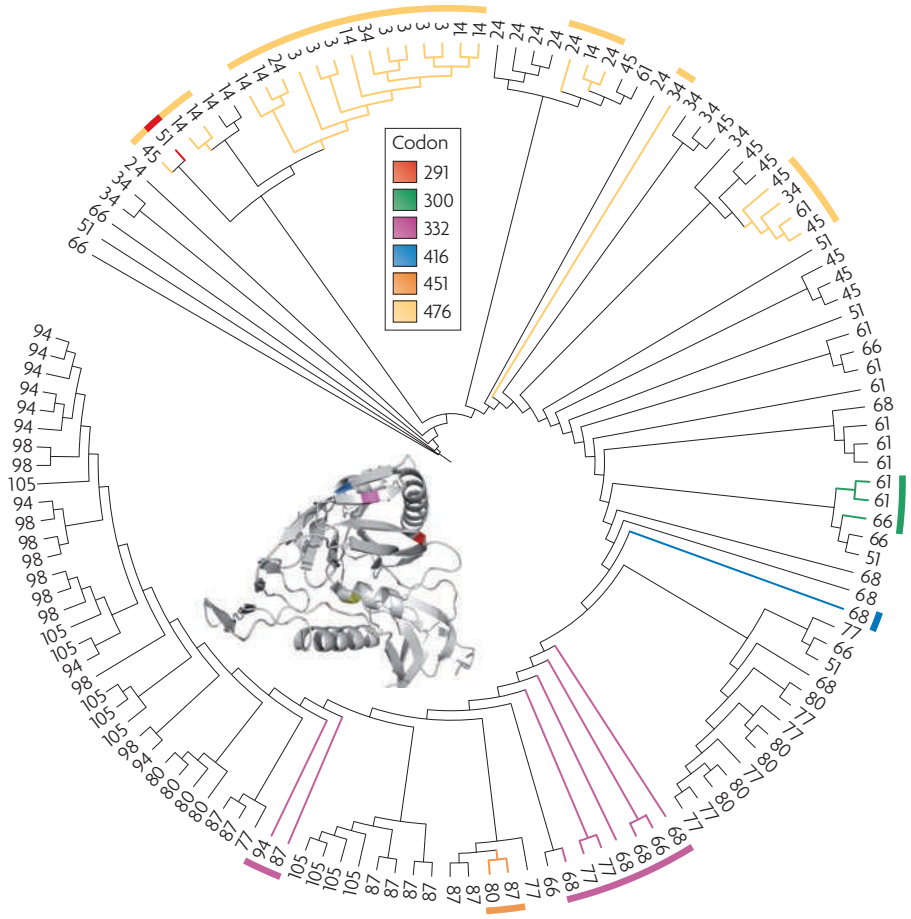

Figure 5 | **Positive selection acts episodically on different codons in the envelope protein of HIV.** The leaves of the phylogenetic tree shown here represent coding regions of the Env glycoprotein (gp120) of a single patient (patient 1 from REF. 86) isolated at different time points after seroconversion, that is, after the patient became HIV-positive. The numbers on each branch of the tree indicate the time post-seroconversion (in months). Colours reflect codons that are under positive selection on coloured branches of this tree. They correspond to the colours on the molecular structure of gp120 (REF. 111), which is shown to the left of the centre. Specifically, six codons are highlighted whose evolution follows an episodic pattern. For instance, codon 291 (red) is under positive selection in a sequence isolated 51 months post-seroconversion, but not in any other sequences. Codon number 300 (green) is under positive selection in three sequences isolated at 61 and 66 months post-seroconversion, but in no other sequences. Codon numbers reflect coordinates relative to the beginning of the gp120 sequence from REF. 111. Episodic selection was detected with a maximum-likelihood codon-based approach[85]. Branch lengths do not reflect the amount of sequence change along each branch.

Although most of the information available for the study of molecular evolution comes from molecules, it is worth pointing out that similar epistatic phenomena also exist on higher levels of organization. Metabolic networks are a case in point. Here, many individual mutations that eliminate enzyme-coding genes have very little effect on cell growth[76–79]. Nonetheless, pairs of mutations often have strong effects, indicating epistasis. These effects are not necessarily detrimental, as multiple deletions of metabolic genes can lead to increased growth[80].

Shifting foci of selection. A third class of prediction from the model of FIG. 3 is that at different points in time, different parts of a molecule should be subject to positive selection: residues that are subject to positive selection at some time may evolve neutrally at other times. Molecular data and phylogenetic methods are now sufficiently rich to test such predictions, most notably owing to the advent of phylogenetic methods based on maximum-likelihood estimation that allow detection of positive selection that is not specific to individual branches of a phylogenetic tree or to individual codons in a protein[81–85].

Several molecular evolution studies support the above prediction. For example, different residues of the influenza haemagglutinin antigen are associated with different antigenic properties[26,27]. However, most amino-acid sites associated with changes in antigenic properties, and thus probably subject to positive selection, evolve neutrally in other viral lineages[27]. Similar patterns can be observed in the evolution of the HIV envelope glycoprotein Env[85,86]. A maximum-likelihood model that allows individual codons to shift between a state of neutral evolution and positive selection detects that such shifts are frequent[85]. As an example, consider the phylogenetic tree in FIG. 5, which is based on the evolution of the Env coding sequence in an HIV-positive individual over 10 years. Selection at individual codons is episodic — it occurs on some branches of the tree but not on others. In addition, codons that are under selection along some branches evolve neutrally along others. In some proteins, such as cytochrome *b*, as many as 95% of amino-acid sites can be subject to a selection pressure that varies over time[87,88].

In summary, three lines of evidence support the notion that neutral or deleterious change can alternate with beneficial change at any one position of an evolving molecule. These are patterns of episodic diversification among evolving molecules, of pervasive epistatic interactions among mutations and of shifting foci of positive selection. Such an alternation of different kinds of change is necessary if a mutation that was neutral at the time of its origin is to turn into a beneficial mutation later, once other parts of the molecule have changed, and vice versa. This evolutionary scenario, in turn, can explain how neutral mutations can be crucial for functional innovation, yet need not remain neutral forever.

Limitations of the model. Because the model of FIG. 3 is a highly abstract representation of a complex real-life process, a few caveats are in order. First, we do not know whether individual mutations are ever exactly neutral, because we cannot measure the effect of very weak mutations in the laboratory[89]. However, because mutations with fitness effects smaller than the inverse of the effective population size[1] are invisible to selection, exact neutrality is not required. Even successive combinations of slightly deleterious and compensatory beneficial mutations can lead to

almost neutral evolutionary dynamics[69], and large populations might facilitate such combinations[62,90].

Second, beneficial mutations might not occur singly but often in bursts, a situation in which an initial mutation triggers opportunities for further beneficial mutations to occur. For example, only one of more than 10 transitions between haemagglutinin antigen clusters that share key antigenic properties is associated with a single amino-acid substitution. Each of the other cluster transitions are associated with multiple substitutions[26]. Nonetheless, periods of adaptive evolution can alternate with neutral diversification even in cases in which multiple beneficial mutations occur in bursts[27].

Third, the model does not concern evolution in a fluctuating environment, in which molecules might be subject to changing selection pressures[9]. This simplification is necessary to highlight that, even in a constant environment, the fitness effects of individual genetic changes can vary dramatically over time. Environmental fluctuations might contribute further to such changing fitness effects.

Summary and outlook

The perspective presented here follows from two observations. First, genotype space is partitioned into multiple genotype networks associated with different phenotypes. Second, neutral genetic change can become beneficial or detrimental, depending on other changes that occur after it arose. For beneficial mutations that represent transitions between genotype networks (FIG. 3), the second observation emerges naturally from the first. The resulting view can support the importance of neutral mutations for evolutionary innovation, while affirming that selection is key to the explanation of patterns of observed genetic variation. This view can thus reconcile neutralism and selectionism.

This perspective is simple, and its elements have precedents in earlier work[91–94]. However, it becomes powerful only when we consider complex molecular phenotypes, in which this perspective emerges naturally from the organization of phenotypes in genotype space. This knowledge has not been available until recently. For instance, although discussions of epistasis have permeated the evolutionary literature for decades, the meaning of epistasis and the limitations of the word neutrality become clearest in the context of molecular phenotypes (FIG. 4). In addition, molecular engineering experiments demonstrating the importance of neutral change need technology that was unavailable until recently.

Two complementary research programmes are suggested by this perspective. The first regards the interconversion of neutral, beneficial and deleterious mutations. At what rate does such interconversion occur? How does it depend on the molecule studied? Does the relative frequency of neutral versus beneficial change vary among molecules? Phylogenetic analysis tools to answer these questions are within reach. Second, laboratory evolution experiments tend to focus on mutations with large effects on molecules. If one wants to rapidly engineer proteins with new functions, it is the rational thing to do. However, as a result, such laboratory studies might be subject to an ascertainment bias[74]: they might not detect the mutations of weak effects that prepare the ground for new molecular functions. Experimental techniques that give us an unbiased view on these mutations are needed to assess their incidence and importance relative to mutations that are immediately subject to strong selection. These two research programmes would turn a fundamental conceptual tension into a constructive research effort. This effort can elucidate exactly where on the spectrum between extreme neutralism and extreme selectionism biological evolution unfolds.

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FURTHER INFORMATION

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