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The role of mutational robustness in RNA virus evolution

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Abstract

RNA viruses face dynamic environments and are masters at adaptation. During their short 'lifespans', they must surmount multiple physical, anatomical and immunological challenges. Central to their adaptative capacity is the enormous genetic diversity that characterizes RNA virus populations. Although genetic diversity increases the rate of adaptive evolution, low replication fidelity can present a risk because excess mutations can lead to population extinction. In this Review, we discuss the strategies used by RNA viruses to deal with the increased mutational load and consider how this mutational robustness might influence viral evolution and pathogenesis.

RNA viruses exhibit extremely high mutation rates, orders of magnitude greater than those of most DNA-based life forms¹ (BOX 1). Although the measurement of viral mutation rates is a complex issue in itself, the studies carried out to date suggest that many RNA viruses generate 10^{-4} to 10^{-6} errors per nucleotide, which is equivalent to approximately one mutation per genome, per replication cycle². Given the large population sizes observed in both experimental and natural infections with these viruses, every possible point mutation and many double-mutation combinations could theoretically be generated during each replication cycle within a population. Even a defined molecular clone quickly transforms into a collection of related sequences when introduced into cells¹. This low replicative fidelity ensures that viral populations can generate and maintain mutations that allow them to quickly adapt to changes in the environment. The mutability and fleeting existence of each viral genome means that RNA virus populations exist as dynamic mutant networks in which sequences are continuously diversified and regenerated by mutation of related

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The authors declare no competing financial interests.

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sequences (FIG. 1). The low replicative fidelity seems to be crucial for viral survival in the host ecosystem, as variants with abnormally low mutation rates are attenuated *in vivo*³⁻⁵.

The focus on mutation as a driving force in viral evolution has tended to overlook the tremendous cost of low replicative fidelity. Most mutations have deleterious effects on viral fitness. In vesicular stomatitis virus (VSV), more than 90% of random single-nucleotide mutations reduce replicative fitness, and 40% are lethal⁶. Similar trends have been found in tobacco etch virus and the phages ΦX174 and QB⁷. Furthermore, increasing error rates pharmacologically, with mutagenic nucleoside drugs^{8,9}, or genetically, through the use of variant RNA-dependent RNA polymerases¹⁰⁻¹², leads to viral extinction. These studies indicate that the mutation rate in RNA virus populations is perilously close to the maximum tolerable error rate. The mutational tolerance of a virus will determine the type (for example, variation in structural or non-structural proteins) and extent of genetic diversity that can be maintained in the population. Thus, viral population diversity results from both the generation of and the tolerance to mutations; these two factors together drive adaptation and viral evolution.

It has long been recognized that not all genotypic changes are expressed as alterations in phenotype, and in population genetics, this buffering of mutational effects is termed genetic robustness. Early work on genetic robustness was largely based on theory (reviewed in REF. 13), but a number of experimental studies over the past 10 years have established and extended the concept of genetic robustness and shown that this buffering allows a viral population to increase its genetic diversity without a dramatic alteration in phenotype. Importantly, these experimental systems have also begun to elucidate the molecular underpinnings of mutational tolerance and to identify the conditions in which genetic robustness is adaptive. Recent studies further suggest that the relationship between robustness and evolvability might be particularly important for viral pathogenesis¹⁴.

As a result of this recent work, we now have a clearer picture of how robustness influences the short- and long-term evolution of RNA viruses. In this Review, we begin by defining genetic robustness and how it can be measured, before considering how genetic robustness influences the composition of viral populations. We then discuss the mechanisms that contribute to genetic robustness and how they relate to viral pathogenesis, and finally consider the impact of genetic robustness on antiviral therapeutic strategies.

Robustness: defined and measured

de Visser and colleagues have provided a useful working definition of robustness: “Robustness is the invariance of phenotypes in the face of perturbation” (REF. 13). They further classify robustness on the basis of the source of perturbation, which can be either genetic or environmental. Environmental robustness refers to the stability of traits across different environments (for example, particular habitats or conditions, such as heat shock). Genetic robustness describes scenarios in which the perturbation (in other words, the mutation or mutations) is heritable. Conceptually, genetic robustness is a form of epistatic interaction; the degree to which genetic variation is expressed depends on the genetic background. Epistatic interactions among genes determine the phenotypic expression of mutant alleles, and if the collective effect of these interactions results in maintaining the phenotype, then this buffering manifests as robustness. Although the virology literature often describes mutational rather than genetic robustness, both terms refer to the same phenomenon. We use the term mutational robustness in our discussion below.

The simplest measure of mutational robustness is to quantify the mutational fitness effect of individual mutations. The mutational fitness effect has been determined in a number of

viruses by introducing random point mutations into the viral genome and measuring their effects on replicative efficiency in competition assays^{6,15,16}. Viruses that exhibit a small average mutational fitness effect are considered mutationally robust, whereas those exhibiting a large mutational fitness effect are considered fragile (or brittle). A less controlled, but equally valid, measure of robustness is to quantify the sensitivity of the virus to nucleoside analogues. Many of these nucleosides are misincorporated into viral genomes during replication and increase the observed mutation rate by templating mismatches^{14,17,18}. These studies do not provide an absolute measure of robustness, given that each study focuses on a single virus or strain. Many discussions of the topic divide robustness into categories according to the evolutionary or mechanistic origins of the robustness^{13,19}. For virologists, it is perhaps more useful to discuss robustness in the context of viral genome structure, replication and fitness. Below, we discuss the main factors that influence viral robustness.

Population size

RNA viruses have a tremendous reproductive capacity, generating thousands of progeny per genome. Despite frequent bottleneck events, a total population of millions of viruses in an infected host is not uncommon. Evolutionary theory suggests that a ‘safety in numbers’ phenomenon is at play, whereby mutational robustness exists at the population level despite the negative impact of mutation on each individual in the population (reviewed in REF. 19). The efficiency of negative selection is the product of the effective population size (N_e) and the average mutational fitness effect. Thus, in large populations, strong selection will quickly purge mutants with lower fitness, and the most fit sequence will dominate the mutant spectrum, accompanied by very low frequencies of numerous variants with intermediate fitness. This frequency distribution has been observed in host-derived populations of poliovirus¹⁴ and foot-and-mouth disease virus²⁰. Mutational robustness can therefore be achieved by generating a large number of progeny, preserving the most fit sequence and ensuring its survival in a given environment. In this manner, large population sizes preserve the invariance of phenotype in the face of mutational perturbation.

Co-infection

A larger population size can also result in a higher multiplicity of infection such that a single host cell more often supports replication of at least two different viral genomes. In such cases, genetic complementation can increase the robustness of the viral population. This means that although a mutated viral protein can limit or even halt viral replication at a low multiplicity of infection, the defect is masked at a high multiplicity of infection because other genomes in the cell encode a functional version of the defective protein. This contribution to robustness is commonly observed in cell culture systems and explains why phenotypic differences among competing strains are harder to distinguish at high multiplicities of infection (for examples, see REFS 14,21). The importance of complementation to mutational robustness has been demonstrated by subjecting the RNA phage $\Phi 6$ to 300 passages at either a high or a low multiplicity of infection²². At a high multiplicity of infection, complementation was frequent and purifying selection against mutated genomes was reduced. However, because these defective genomes could be propagated, this strategy weakened long-term selection for mutational robustness. By contrast, at a low multiplicity of infection, viral populations evolved to become more mutationally robust.

Many RNA viruses undergo recombination or reassortment during replication. This exchange of genetic information can increase the genetic diversity of the population by combining previously unique mutations into the same genome. Although the impact of viral sex on mutational robustness and viral evolution remains somewhat obscure, it is clear that

this process can also repair mutated genomes (reviewed in REF. 23). Recombination could also disrupt the genetic linkages between beneficial and detrimental mutations, thus reducing the impact of these detrimental mutations on the selection of the beneficial mutations. Given the complex relationship between complementation, recombination and robustness, the effective multiplicity of infection in infected individuals is likely to be a crucial factor influencing viral evolution.

Fitness landscapes and survival of the flattest

The phenotypic stability provided by mutational robustness confers a selective advantage, but is it necessarily adaptive? In principle, mutational robustness could evolve as a convenient side effect of selection for a different phenotype, such as increased inter-host survival or transmissibility¹³. Perhaps the clearest argument in favour of an adaptive basis for mutational robustness is the resultant enhanced tolerance to the excess mutational load caused by a low replicative fidelity, as this tolerance suggests that high mutation rates probably select for robust genomes.

One way to illustrate robustness is using fitness landscapes, which represent the relationship between genotypes and fitness (FIG. 2). The 'altitude' at any given location is the fitness associated with that particular genotype. The 'ground level' is a representation of the range of genotypes in the sequence space. Two sequences that differ by a single mutation are neighbouring points at the ground level, whereas highly divergent sequences are well separated. The environment and its selective pressures determine the contours of the landscape. Fit but mutationally brittle populations occupy steep peaks, whereas robust populations reside on broader hills. When the mutation rate is high, the populations are pushed away from the fitness peaks and out into the surrounding sequence space lower down the slopes. Selection favours robust populations, as they are better able to accommodate mutations without a change in fitness (outward movement from the fitness peak corresponds to movement down a gentler slope). In experimental systems, it has been difficult to distinguish fitness from robustness (see below).

This process, whereby populations buffer the negative effects of mutation by migrating to regions of sequence space corresponding to flatter, selectively neutral fitness landscapes, is termed survival of the flattest. A pioneering *in silico* study used self-replicating digital organisms to show that selection does indeed favour slowly replicating, robust populations over their fitter, more fragile counterparts²⁴. The first evidence for survival of the flattest in a biological system came from studies of RNA viroids in plants²⁵. A viroid strain with a slightly larger neutral neighbourhood was able to outcompete another strain with a faster replication rate (a fitter strain) when these strains were propagated in a mutagenic environment (plants exposed to ultraviolet light). A similar study using two distinct populations of VSV found that selection favoured the slower replicating, mutationally robust population over a faster replicating population when the two populations were competed in the presence of mutagenic drugs²⁶.

Both experimental studies clearly demonstrate survival of the flattest^{25,26}, but the distinction between the fittest and flattest might be an artificial one. In theoretical discussions, fitness and robustness are often considered separately for the sake of argument and to illustrate that flatter populations can, in principle, outcompete fitter but more brittle ones (FIG. 2). This is not necessarily the whole story; a population can be both fit and robust, occupying a high, broad peak. Measurements from competition assays cannot distinguish between replicative fitness and mutational robustness, as both determine the number of successful progeny over multiple passages²⁴. In a competition assay between two populations, a given population could dominate either by replicating faster (which is the most commonly used parameter for

measuring fitness in the laboratory) or by producing progeny with preserved fitness. The latter outcome would be due to robustness, whereas the former scenario would indicate only that the population was dominant because it replicated faster. Perhaps this is the reason that selection for mutational robustness can be demonstrated in competition assays only at above-normal mutation rates, when the beneficial effect of increased mutational tolerance outweighs any reduction in replicative efficiency. This issue was addressed by comparing wild-type poliovirus to two poliovirus variants that contain a large number of synonymous mutations^{14,27,28}. All three viruses have the same consensus amino acid sequence and exhibit similar replication kinetics. However, the three viral populations are genetically distinct and occupy unique fitness landscapes. One of the mutant populations was found to be less mutationally robust than the wild-type virus and also less fit in competition assays. The simplest interpretation of these data is that differences in the number of viable progeny generated during each round of replication drive the observed fitness differences. Furthermore, these data suggest that mutational robustness is an important component of viral fitness, buffering the negative effects of mutation even at basal RNA virus mutation rates.

Mechanisms of robustness at the genome level

The identification of VSV and poliovirus populations that differ in their mutational tolerance suggests that robustness can be defined at the level of the viral genome. In many RNA viruses, the RNA genome contains secondary and tertiary structures that are important for replication, packaging and other key functions. The robustness of such structures to mutation has been extensively studied in tRNA, ribozymes and other small RNA molecules for which the thermodynamics of folding is easily simulated. Early folding simulations of tRNA demonstrated that a large set of sequences (genotypes) could assemble into similar shapes (phenotypes)^{29,30}. Because these diverse sequences are selectively neutral and connected through mutation, this type of genotype-to-phenotype map is often called a neutral network³¹. More recently, cycles of mutagenesis and selection have been used to create genotypically diverse ribozyme populations that retain their ability to cleave a phosphodiester bond in an RNA oligonucleotide³². The accumulated cryptic variation did not affect the phenotype and was selectively neutral in a given environment (mutationally robust), but because the mutations potentially provide a reservoir of beneficial mutations for future environmental changes, these ribozyme populations could also be environmentally robust. In RNA viruses, structures that regulate replication or translation are highly conserved. An *in silico* experiment with HIV, hepatitis C virus and dengue virus compared these conserved, functional elements to non-conserved RNA structures from the same species and found that the conserved functional elements were more mutationally robust than their non-conserved counterparts³³. Similar phenomena have been documented in viroid structures^{34,35}.

These data highlight the link between robustness and the degeneracy of RNA structural elements; multiple genotypes can give rise to the same phenotype. The genetic code is also degenerate, and nearly all amino acids are encoded by more than one codon. However, given the nature of the genetic code, synonymous codons will yield distinct amino acid changes when they are mutated (FIG. 3). For example, although both AGG and CGG code for arginine, the frequencies of mutations that give rise to non-synonymous codons are 78% and 56%, respectively, for these two synonymous codons. Furthermore, the amino acids introduced by single point mutations will also differ in their biochemical properties (such as hydrophobicity, polarity and charge). Codon usage can therefore determine how mutations are tolerated at the protein level. Bioinformatic studies of several RNA viruses indicate that the highly variable surface epitopes of viral proteins might be enriched for volatile codons that tend to mutate non-synonymously^{36,37}. This is in contrast to more conserved domains,

which contain codons with a lower predicted rate of non-synonymous substitutions. Theoretical studies have also highlighted the potential importance of codon volatility in rapidly evolving protein domains³⁸.

Codon choice has been proposed to be an important mechanism for robustness in RNA viruses¹⁴. In a recent study, wild-type poliovirus was compared to two variants with a large number of synonymous mutations¹⁴. These variants, Max and SD, were created by *de novo* gene synthesis and contain 566 and 934 substitutions, respectively, in the 2,643-nucleotide sequence encoding the viral capsid protein^{27,28}. Because these variants were designed as negative controls for studies of codon bias and viral translation, their GC content, predicted free energy of RNA folding and codon usage frequency across all 64 codons were nearly identical to those of the wild-type virus. In the new study, the viral populations derived from replication of these three viruses were each found to contain a unique set of polymorphic amino acid substitutions in the capsid protein, and there was lower sequence similarity in the capsid sequence relative to the rest of the genome among the three viral strains¹⁴. The SD population was less mutationally robust than either the wild-type or Max populations, suggesting a role for codon choice in mutational tolerance. Interestingly, the synonymous substitutions in the Max variant were based on altering local codon pair bias, whereas the codons in the SD variant were randomly shuffled relative to the wild-type sequence. Perhaps the more drastic reorganization of the capsid sequence in the SD variant disrupted local patterns of codon volatility in the capsid proteins, thereby increasing the frequency of non-synonymous substitutions in structurally conserved domains.

Chaperones: cellular modulators of robustness

Another way to maintain the functionality of proteins carrying destabilizing mutations is through the activity of molecular chaperones³⁹. Mutations that accumulate during viral replication can destabilize viral proteins and increase their tendency to misfold and aggregate. Because they face such a high mutation load, RNA viruses are likely to be highly dependent on the action of host chaperones and the quality control machinery to buffer mutational effects and to maintain the functionality of the viral proteome. Molecular chaperones are central regulators of the conformation and life cycle of proteins in the cell; these chaperones are fundamental for protein biogenesis, as they promote folding and assembly of newly translated polypeptides and assist their trafficking to organelles. Chaperones also monitor destabilized proteins, preventing their aggregation and promoting quality control through either refolding or facilitating the degradation of misfolded proteins. Accordingly, chaperones can preserve the functionality of proteins that have been destabilized by mutations, and thereby buffer detrimental mutations³⁹. Most viruses use cellular chaperones during their life cycle both to solve their own protein-folding problems and to interfere with cellular processes, such as signal transduction^{40,41}. Many examples of the interaction between cellular chaperones and viral factors at different stages of the infectious cycle have been reported in recent years⁴²⁻⁵².

Although the role of chaperones as extrinsic modulators of viral diversity has not been systematically explored, some examples are beginning to emerge. For instance, the capsid precursor (P1) proteins of several picornaviruses, including poliovirus, rhinovirus and coxsackievirus, require heat shock protein 90 (HSP90) for folding and assembly⁴¹. Experiments in poliovirus showed that P1 uses HSP90 to fold into a conformation that is competent for processing by a viral protease; this processing is required for subsequent assembly of mature capsids⁴¹. Deep-sequencing analyses indicate that pharmacological inhibition of HSP90 results in a profound alteration of the mutation distribution in the viral population (R. Geller, A. Acevedo, J.F. and R.A., unpublished observations). Viral populations grown in HSP90-inhibited cells harboured substantially fewer amino acid

substitutions (36% reduction) than viral populations grown in wild-type cells, suggesting that HSP90 enables expansion of the viable protein-coding sequence space in viral populations. Whether different chaperones preferentially buffer specific types of mutation remains to be determined, but if this were to be the case, it would imply that chaperone levels influence the evolutionary trajectory of the viral proteins in a given cell. In addition, the role of the quality control and degradation machineries, such as the ubiquitin–proteasome pathway, in purging dominant-negative or otherwise deleterious mutations from virus-infected cells is also relevant, but has not yet been studied. This function might be particularly important in the case of multisubunit complexes, such as the capsid, for which incorporation of misfolded subunits could create defective viral particles⁵³.

Given the importance of chaperones to viral replication and robustness, it is not surprising that viral infection often elicits cellular stress responses that lead to enhanced expression of chaperones^{40,54}. In principle, these responses could be triggered by the production of mutated, destabilized viral proteins during replication, as well as by the enhanced load put on the cellular biogenesis machinery by rapidly replicating viruses. It is intriguing to speculate that viral activation of cellular stress signalling pathways increases the availability of chaperones and other extrinsic modulators of robustness.

Robustness and evolvability

Our discussion thus far has focused largely on how mutational robustness buffers the negative impact of high viral mutation rates. However, the selective neutrality, or flatness, of the viral fitness landscape will also define the set of mutations that are viable and potentially adaptive. A key unresolved question, then, is whether mutational robustness increases or decreases evolvability, which we refer to as the capacity of a virus to increase in fitness through adaptation¹³. According to Fisher's fundamental theorem, "The rate of increase in fitness of any organism at any time is equal to its genetic variance in fitness at that time." (REF. 55.) Because robustness expands the neutral network of genotypes in a population, it should reduce phenotypic variation and the efficiency of natural selection. However, robustness will also increase the amount of cryptic genetic variation in a population, and these neutral mutations might have epistatic interactions with subsequent mutations, thus increasing the range of adaptive phenotypes that are available in the local sequence space. This pro-evolvability model is supported by thermodynamic studies of RNA folding^{29,30,56}, and recent theoretical work suggests that robustness favours adaptive evolution when the number of phenotypes accessible to an individual through mutation is smaller than the total number of phenotypes in a given landscape⁵⁷.

Experimental evidence from a number of systems also links robustness to increased evolvability. Mutagenesis studies with the cytochrome P450 system suggest that thermostability determines mutational robustness and the evolutionary capacity of proteins⁵⁸. Perhaps the most intriguing results come from the recent studies of thermostable ribozymes³². As described above, in mutationally robust ribozyme populations that retained function, the cryptic variation was selectively neutral in the native environment. However, the accumulated diversity was nevertheless beneficial, as the robust populations adapted to a new substrate more rapidly than their less diverse ancestors. Therefore, mutational robustness allows a population to explore a range of genotypes that are neutral in one environment but potentially beneficial in another.

Work in the phage $\Phi 6$ model also suggests a positive correlation between robustness and evolvability. Using derived phage clones that were created by longterm passage²² and varied in mutational robustness, the authors of this study asked whether the clones differed in adaptation to a defined selective pressure⁵⁹. Twelve clones from each lineage were

subjected to intermittent heat shock for 50 additional generations, and the percentage of clones from each lineage that survived heat shock was used to quantify adaptation to this new environment. The mutationally robust strains demonstrated higher survival rates than the less robust strains, indicating that robustness accelerated the fitness gain over the preceding 50 generations. Similar to the ribozyme experiment, this work supports a model in which robustness allows for a greater exploration of sequence space, reducing the number of additional mutations required for fitness gain in a new environment.

This pattern of neutral network exploration and rapid adaptation has also been defined in a phylodynamic study of influenza A virus⁶⁰. In this analysis, the linkage between genotypes and the phenotypes of the surface haemagglutinin (HA) was established using existing sequence databases and antigenic maps, which quantify cross-immunity to different epitopes. Influenza A virus H3N2 isolates were found to move throughout large neutral networks, accumulating genetic variation. This phenotypic stasis is punctuated by sudden changes in phenotype, in which the virus adopts a new antigenic structure. The viral dynamics are triggered by shifts in the host immune environment, aggregated across populations. Interestingly, these epochal shifts coincide with peaks in infections and are well described by epidemiological models of susceptible, infected and recovered individuals.

Evolvability and pathogenesis

Experimental measures of robustness and evolvability require controlled environments with defined selective pressures. By contrast, in nature, viral populations will encounter a range of environmental conditions over their lifespan (FIG. 4). Shifts in host tropism, immune pressure and cellular milieu create a highly dynamic fitness landscape to which the virus must adapt. Because optimal fitness in each environment is determined by a specific combination of mutations, it might be advantageous for the viral population to maintain an assortment of pre-adapted, or exapted, variants that are fewer mutational steps away from a fitness optimum^{32,61}. In this model, mutational robustness would be a selective advantage, as it allows a greater exploration of the sequence space and a more rapid fitness gain.

This hypothesis was tested using the synonymous poliovirus variants described above¹⁴. Although the wild-type virus and both the SD and Max mutants all encode the same consensus amino acid sequence across the capsid, each virus was found to have a unique spectrum of low-frequency variants within the population. That is, the three populations had different cryptic genetic variation. Furthermore, the less mutationally robust SD population was attenuated relative to the wild type in a mouse pathogenesis model, whereas the more robust Max population was similar to the wild type in terms of virulence *in vivo*. These data support a model in which robust populations, which are located on the flatter fitness landscapes, are able to maintain sufficient cryptic variation for rapid adaptation to new selective pressures. Alternatively, the SD population might just have the 'wrong' set of variants compared to wild-type poliovirus, and these variants might reduce the ability of the SD population to adapt to the dynamic environment of the infected individual. In this alternative model, robustness would be a surrogate marker for the location of the population in the genetic sequence space. It is difficult to distinguish between these two models because the origin of a population in the sequence space defines the set of available mutations for that population and places it in a fitness landscape. This confounding effect of local sequence space might explain why another study found a negative correlation between robustness and evolvability⁶². In this study, the ability of two divergent VSV populations to gain fitness in a new cellular host was compared, and the less robust population proved to be more adaptable. Studies with larger numbers of viruses and distinct selective pressures will be needed to clarify which of these two models of robustness and evolvability is more generalizable.

Robustness and antiviral therapy

Given the importance of mutational robustness to the behaviour of RNA viruses, does robustness represent an opportunity for, or a barrier to, antiviral therapy? With their high mutation rates, RNA virus populations replicate near the limit of viability (the so-called error threshold). A population can remain at equilibrium despite a high mutation rate^{63,64}; however, small increases in the mutation rate will disrupt this balance, and the population will lose meaningful genetic cohesion in an avalanche of errors. Although such an error catastrophe has not been observed experimentally, early studies of VSV and poliovirus showed that mutagenic nucleoside drugs are lethal to RNA virus populations^{8,65,66}. Recent work on host cell restriction of retroviral infection suggests that some antiviral defence pathways rely on inducing lethal mutagenesis (reviewed in REF. 67). For example, members of the APOBEC family of RNA-editing enzymes deaminate C residues to U residues during reverse transcription of retroviral RNA. As a result, the progeny genomes exhibit G-to-A hypermutation and reduced infectivity^{68,69}. It is important to note, however, that error catastrophe and lethal mutagenesis describe different processes. Bull *et al.* have explained the distinction most clearly: “an error catastrophe is an evolutionary shift in genotype space, whereas extinction is a demographic process, a drop in the absolute abundance of individuals in the population” (REF. 70). This distinction is more than semantic, as a population shift to a more mutationally robust region of genotypic space could delay or even prevent error catastrophe.

These observations have stimulated an interest in using lethal mutagenesis as a broadly effective therapeutic strategy for RNA virus infections (reviewed in REF. 71). Ribavirin and other nucleoside analogues have demonstrated efficacy against poliovirus, lymphocytic choriomeningitis virus, Hantaan virus, Lassa fever virus, foot-and-mouth disease virus and HIV^{66,72–76}. Lethal mutagenesis was initially thought to be resistance-proof, as a newly arising drug resistance mutation would be shackled to a lethal mutation in the same genome. However, it is now clear that a viral polymerase can evolve biochemical resistance to mutagenic nucleosides by either excluding the drug from its active site or lowering its intrinsic error rate^{4,5,77,78}.

Because high mutation rates will select for evolved robustness, RNA viruses could conceivably achieve resistance by moving to flatter, more neutral regions of the fitness landscape. In other words, viruses could adapt to the drug by becoming more tolerant to mutation. This possibility was examined using two closely related enteroviruses; human coxsackievirus B3 was found to be mutationally fragile, exhibiting substantially less genetic diversity and tolerance to non-synonymous mutation than poliovirus¹⁸. Human coxsackievirus B3 was also more susceptible to ribavirin-induced lethal mutagenesis. These results demonstrate the importance of relative robustness in determining the therapeutic efficacy of mutagenic drugs across viral species. In a separate study, the potential for the emergence of drug resistance through evolved robustness was examined directly by exposing lymphocytic choriomeningitis virus populations to 5-fluorouracil (5-FU) mutagenesis for nine passages. Both the evolved populations and their ancestral clones remained susceptible to lethal mutagenesis with higher concentrations of 5-FU, suggesting that robustness does not select for resistance⁷³. These results are consistent with a recent theoretical treatment of the topic, in which the benefit of increased robustness was eclipsed by the detrimental effects of higher mutation rates on fitness⁷⁹.

The manipulation of mutational robustness could be used to design strategies to restrict the evolution of live-attenuated viral vaccine strain candidates. The traditional process of attenuation through serial passage, forces a virus to adapt to a foreign host environment. The accumulated mutations move the population to a new region of sequence space that is

unable to support efficient replication and spread in the native host. Studies have shown that small changes in the error rate of an RNA virus owing to mutations in the viral RNA-dependent RNA polymerase gene can substantially reduce population diversity, resulting in attenuation^{4,80}. The study of synonymous poliovirus populations suggests that reorganizing the connectivity of viral mutant networks can achieve the same goal¹⁴. In this case, relocation of a population in sequence space rendered that population less mutationally robust. The ruggedness of the associated fitness landscape can reduce the capacity of the virus to generate fit progeny and adapt to host selective pressures. As others have shown, synonymous mutation has the additional advantages of preserving the antigenic composition of the wild-type virus and reducing the risk of reversion through recombination with homologous sequences in circulating strains^{27,28,81–83}. A rational reduction in mutational robustness might allow for a finer control of evolvability, limiting the ability of a virus to escape immune surveillance and spread in a vaccinated host.

Conclusions and future directions

Mutational robustness is central to the evolution of living organisms. Although genetic variation is the fuel for natural selection, there are limits to the amount of variation that a population can tolerate without loss of fitness or viability. This is particularly true for RNA viruses, which have compact, tightly organized genomes and extremely high mutation rates. It is perhaps not surprising, then, to find that RNA viruses have developed mechanisms to cope with an increased mutational load. What it is more unexpected is the observation that robustness and cryptic genetic variation can have such crucial roles in the rapid and effective adaptation to dynamic environments. In view of the overall paucity of experimental work in this area, it is a particularly exciting time for the field, and there are currently many more questions than answers.

The precise nature of the robustness mechanisms is far from well defined at the moment. The relationship between the genetic structure of a given population and its phenotypic landscape is also unclear. Although many theoretical frameworks have been developed to delineate the relationship between robustness and adaptation, more experimental work is urgently needed to better define how viruses deal with their high mutation rates in the real world and to reveal the molecular mechanisms that underlie robustness at both the RNA and protein levels. Accordingly, it will be important to accurately define the mutation distribution of a viral population in order to establish the proportion of neutral mutations. This measurement would, in turn, serve as a baseline to better define whether robustness does indeed facilitate the increased diversity of viral populations, thereby providing a reservoir of mutations that could allow rapid adaptation to changes in the environment. It will also be of interest to understand how viral manipulation of the host protein and RNA homeostasis machineries, for instance through the induction of stress responses, promotes tolerance to mutations. Although the available data suggest that there are conditions which seem to favour robust populations, we still cannot say for certain whether viruses have evolved to become mutationally robust. We expect that the experimental dissection of robustness across RNA virus taxa will reveal important differences that are linked to viral phenotypes. For example, does robustness allow for the greater exploration of sequence space observed in chronic HIV and hepatitis C virus infection? Does the relative robustness determine which viruses are better targeted by lethal mutagenesis? Defining the evolutionary pay-off or disadvantage of mutational robustness is important because these factors could determine the evolutionary trajectory of viruses in their natural hosts and might hold the key to new antiviral strategies.

Glossary

Fitness	The ability of an entity to survive and reproduce. In experimental virology, replicative efficiency is often used as a surrogate for fitness. In this Review, we define viral fitness as the capacity of a virus to generate infectious progeny
Evolvability	The capacity of a virus or organism with a particular genotype to gain fitness over time after evolving in a given environment
Epistatic interaction	An interaction between mutations such that their combined effect on fitness is different to that expected from their effects in isolation
Mutational fitness effect	The effects of mutations on fitness; often described in a model that combines both the strength and distribution of these effects
Bottleneck	In genetics a dramatic reduction in the number of individuals that can reproduce. Bottlenecks reduce genetic variation and are not necessarily selective events
Negative selection	The removal of deleterious alleles from a population by natural selection. Also called purifying selection
Effective population size	The size of an idealized population that would experience genetic drift in the same way as the actual population. The effective population size (N_e) is often smaller than the total population size
Multiplicity of infection	In virology, the ratio of infectious particles to target cells
Complementation	In the context of this Review the process by which a defective virus can take advantage of functional nucleic acid sequences or proteins from another virus that is infecting the same cell. As a result, the defective virus does not experience loss of fitness from its mutation (or mutations)
Viral sex	The process by which genetic information is exchanged between two different strands of viral nucleic acid. In RNA viruses, this occurs most commonly through switching the replicative template (recombination) or through the exchange of genomic segments (reassortment)
Fitness landscapes	Spatial models that link fitness values to individual sequences
Sequence space	All possible mutations and combinations of mutations present in a given DNA or amino acid sequence
Digital organisms	Self-replicating computer programs that mutate and evolve, often in competition with each other for CPU (central processing unit) cycles
Synonymous mutations	Codon mutations that do not alter the amino acid specificity of the codons. By contrast, non-synonymous mutations do change the encoded amino acid
Volatile codons	Codons with a propensity to mutate non-synonymously, as opposed to synonymously

Codon bias	A difference in the observed frequencies of synonymous codons in a given set of sequences
Codon pair bias	A difference in the observed frequencies of 6-nucleotide codon pairs in a given set of sequences
Phylogenetic study	A study that develops a quantitative model, incorporating both a pathogen phylogeny and epidemiological or immunological data, to describe an infectious disease
Error catastrophe	The loss of meaningful genetic information when a population is pushed beyond its maximum mutation rate. In theoretical models, the error catastrophe has been compared to a chemical phase transition, and a true error catastrophe has not been observed experimentally
Lethal mutagenesis	The process whereby the number of viable individuals, or viruses, in a population is reduced through increases in the mutation rate

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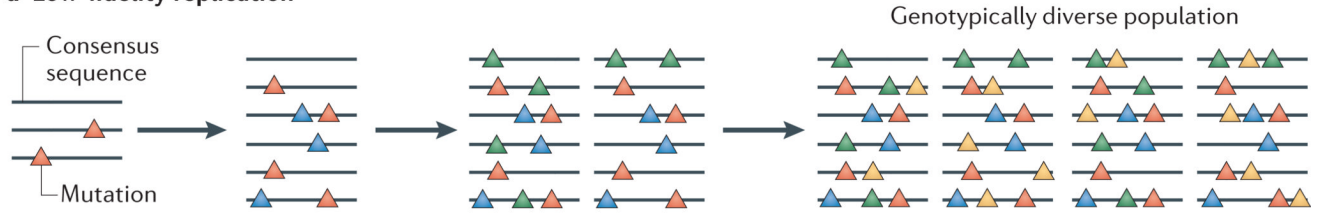
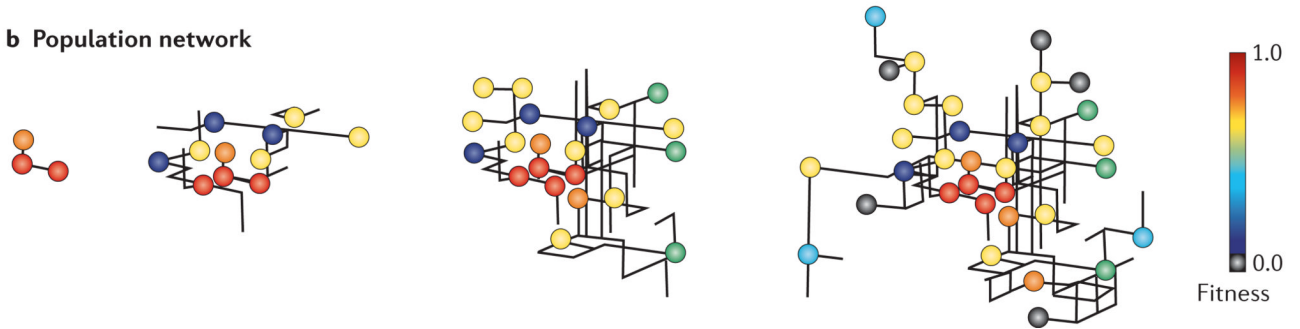
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Box 1**Mutation rates of RNA viruses and quasispecies theory**

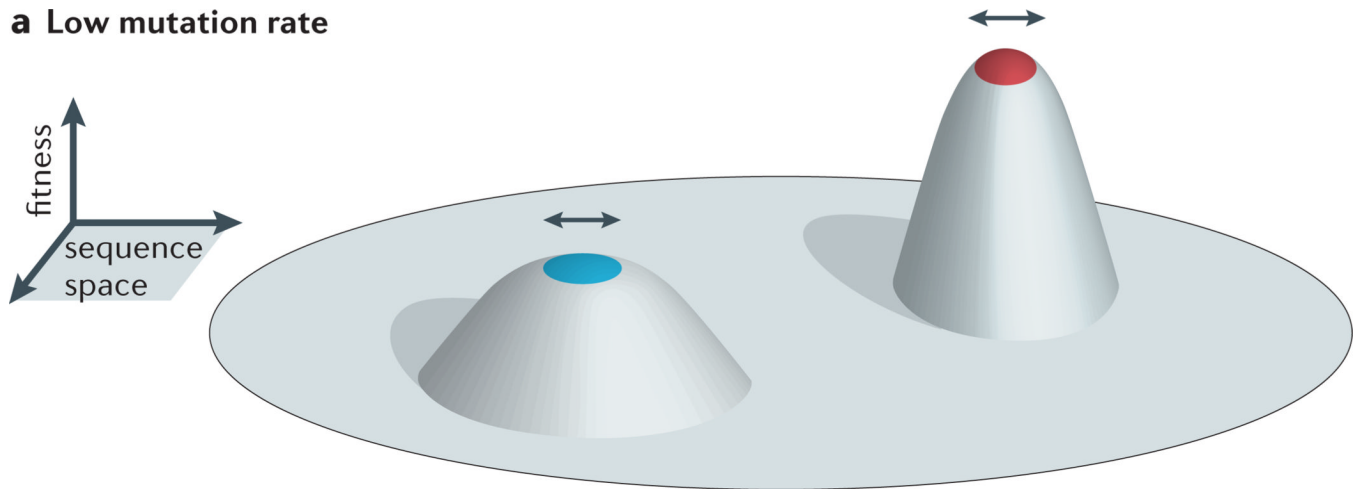
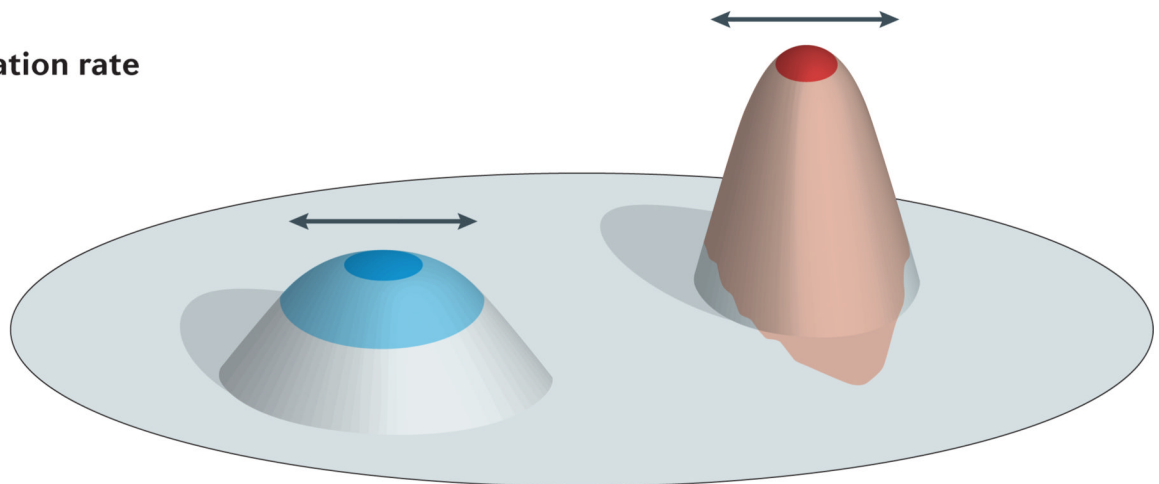
A quasispecies refers to a population of genetically related viruses that are closely distributed around a consensus sequence^{1,84–87}. Such a population is a flexible and diverse group of variants with different replicative capacities and fitnesses. For any given environment, the quasispecies hypothetically consists of a collection of genomes with many, or most, of the possible viable mutations represented. Many of these mutations are lost during multiple replication cycles, as they encode genomes of very low fitness (defined here as the relative capacity to produce infectious progeny) that cannot compete with fitter genomes during replication. However, within each round of replication, new mutations spontaneously emerge or re-emerge at a frequency that is directly determined by the error rate of the viral RNA polymerase.

The mutation rate measures the appearance of spontaneous mutations as a function of time. Observed mutation rates differ among species and also vary across the genome of a given species. This essential parameter is often measured as the number of nucleotide substitutions per base per generation. The mutation rate in unicellular eukaryotes and bacteria is roughly 0.003 mutations per genome per generation⁸⁸. Because RNA viruses are replicated by RNA-dependent RNA polymerases that lack proofreading ability, they have the highest known per bp per generation mutation rates⁸⁹. Double-stranded DNA viruses have mutation rates of between 10^{-6} and 10^{-8} mutations per bp per generation, whereas RNA viruses have mutation rates of between 10^{-4} and 10^{-6} mutations per bp per generation². For comparison, recent genome sequencing studies estimate that the mutation rate of the human genome is $\sim 1.1 \times 10^{-8}$ mutations per bp per generation^{90,91}. Importantly, the estimation of mutation rates described here suffers from a very limited number of experiments and non-standardized sampling of different species and genetic entities. With the use of new sequencing technologies, it is expected that this crucial evolutionary parameter will be better defined in the near future.

There has been considerable debate as to whether the high mutation rate of RNA viruses is adaptive or simply the by-product of selection for other traits such as replicative speed⁹². As detailed in the main text, many newly generated mutations are deleterious, and evolutionary theory suggests that high mutation rates drive selection for mutational robustness¹³.

a Low-fidelity replication**b Population network****Figure 1. Viral populations as mutant networks**

a The consensus sequence (grey line) is the average sequence of a population and might not be represented on any individual genome because of the extremely high genetic diversity of RNA virus populations. Low-fidelity replication, which is a characteristic feature of RNA viruses, results in a diverse population of unique genotypic variants while maintaining the same consensus genome sequence. Mutations acquired in each replication cycle are represented by differently coloured triangles. **b** RNA virus populations can be depicted as networks in which the genetic variants (circles) of varying fitness are connected by mutational pathways (black lines).

a Low mutation rate**b High mutation rate****Figure 2. High mutation rates and survival of the flattest**

In a fitness landscape, the ‘ground level’ is a two-dimensional representation of genotypic sequence space, and the vertical axis gives the fitness value for each genotype or sequence. **a** When the mutation rate is low, populations will be genotypically stable and cluster at the top of the fitness peak. The variant with the highest fitness (red) will easily outcompete all others. **b** When the mutation rate is high, variants spread out over their corresponding peaks. The population on the flatter peak (blue) remains near its fitness optimum and has a higher mean fitness than the population located on the steeper peak (red). The flatter population will therefore prevail in competition with the population on the higher peak. Here, fitness and robustness are contrasted to show the importance of each in determining the dynamics of RNA virus populations. As described in the text, a population can theoretically be both fit and robust and thereby occupy a tall, broad peak. However, the experimental data currently available suggest that fitness and robustness are inversely correlated.

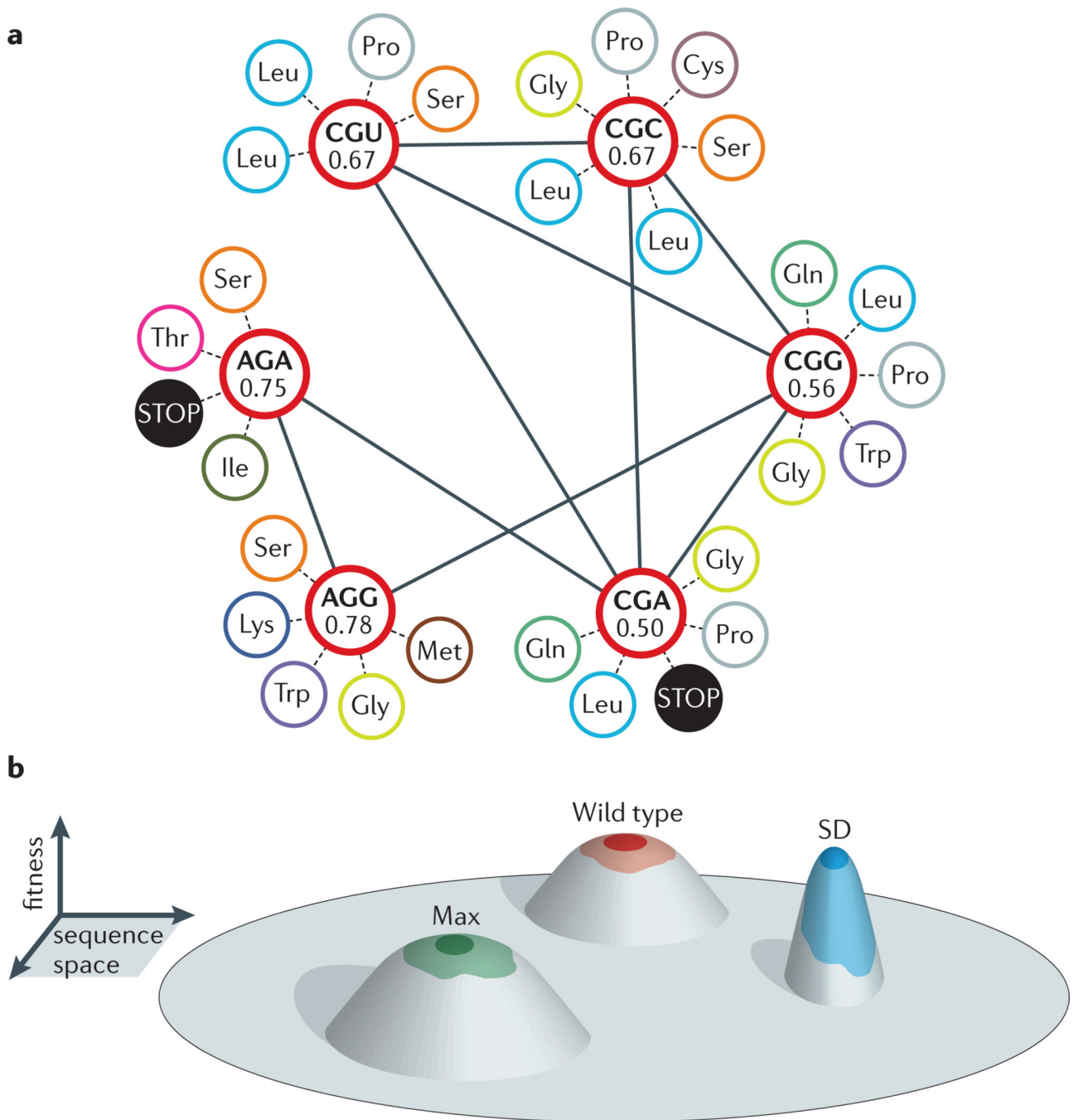


Figure 3. Using synonymous mutation to place populations in distinct fitness landscapes
a A synonymous mutation alters the potential fitness impact of a subsequent mutation. Although two synonymous codons for arginine (such as AGG and CGG) are separated by a neutral A@C mutation, these codons differ in their propensity to mutate non-synonymously and non-conservatively. Shown are all six arginine codons (red circles) and, for each, the proportion of all potential mutations that would be non-synonymous. Synonymous mutations are indicated as solid lines, and non-synonymous mutations are indicated as dashed lines. **b** Large-scale synonymous mutation preserves the consensus amino acid sequence, but relocates viral populations in sequence space. When these viruses replicate

with a high mutation rate, the genetic architecture of the resultant populations differs, and the populations reside in distinct fitness landscapes. The figure shows the results of a poliovirus experiment in which the wild-type virus was compared with two variants, Max and SD, which contain 566 and 934 synonymous substitutions, respectively, in the 2,643-nucleotide sequence encoding the viral capsid protein. The SD variant was found to be the least mutationally robust (for example, it was hypersensitive to an RNA mutagen, relative to the Max variant and the wild type), suggesting that it resides on a steeper, less neutral landscape.

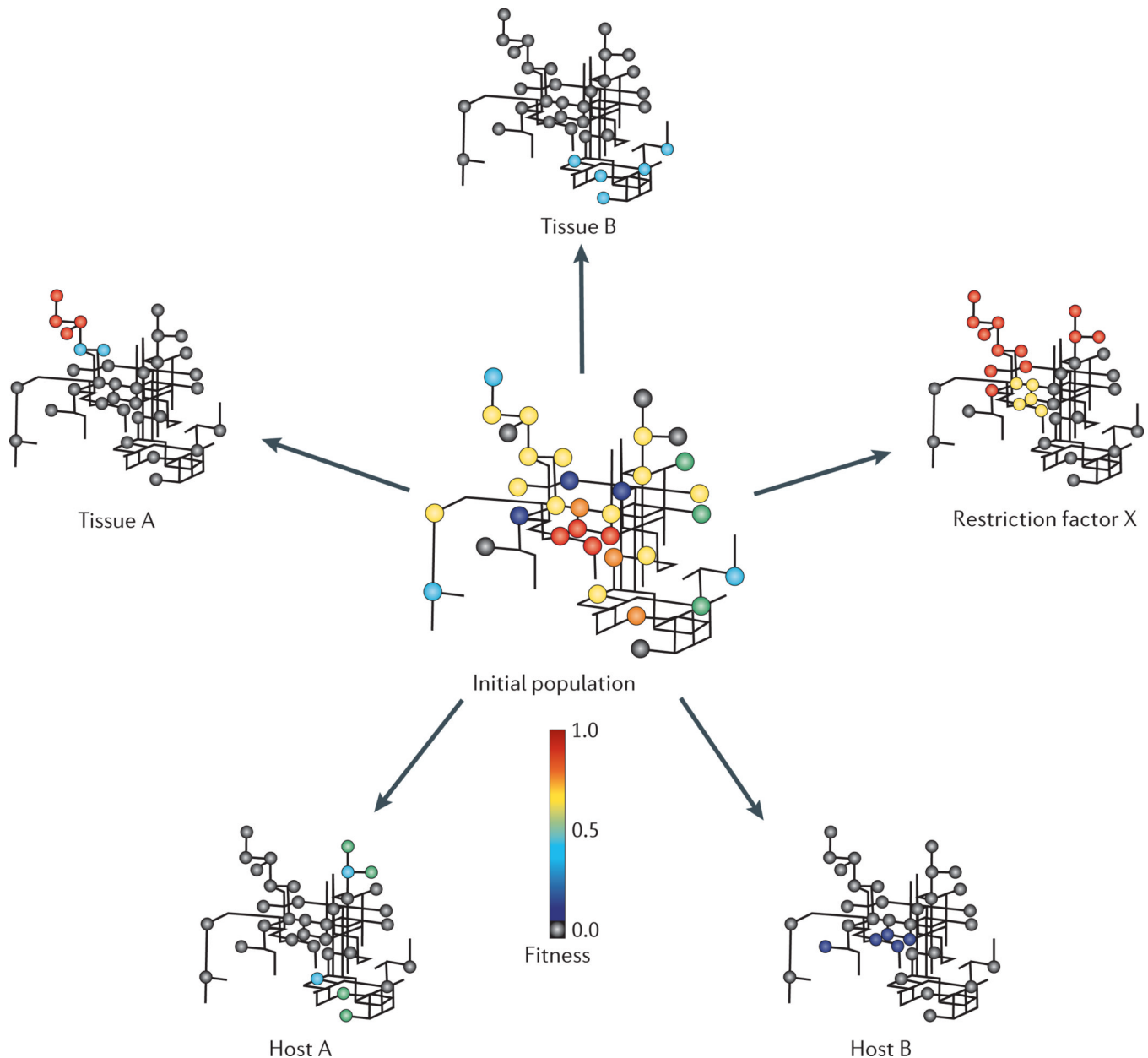


Figure 4. Dynamics of viral networks under selection in the host

The initial viral population is represented by the pipe diagram at the centre of the figure. Each individual variant of the population is represented by a ball, coloured according to fitness, and these balls are connected to each other by a genetic network. Robustness allows the population to explore an extensive region of sequence space, resulting in a larger number of individuals that are able to adapt quickly to environmental challenges. As the viral population confronts changes in the environment, the mutant distribution changes to favour those better adapted to a particular condition, such as replication in a different tissue (tissue A versus tissue B), in a different host (naïve versus immunologically primed) or in a different species (called zoonotic adaptation). Robustness and diversity might also allow the viral population to overcome immunological challenges such as that of antiviral restriction factors. With each of these challenges, the fitness landscape changes and so does the distribution of mutants in the population. The majority of the variants in the initial

population are expected to be poorly adapted to a new environment and to have very low fitness (grey balls). However, given the high degree of phenotypic diversity in the initial founder population, there is a high chance that one or more variants (those with a fitness greater than 0 in the new environment) will quickly adapt and propagate in the new environment, whereas those of low fitness are expected to diminish over time. For example, a variant of intermediate fitness in host A (a green ball) could eventually predominate in this environment if the genotype of this variant is better adapted to this new environment than the other genotypes present in this host. However, if the new environment is host B, then an even less fit variant (dark blue) could theoretically reach fixation as the population is exposed to a different set of selective pressures. The nature of the selective pressure in each new environment is the ultimate factor that decides the distribution of mutants in the population.