The causes of evolvability and their evolution

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Abstract | Evolvability is the ability of a biological system to produce phenotypic variation that is both heritable and adaptive. It has long been the subject of anecdotal observations and theoretical work. In recent years, however, the molecular causes of evolvability have been an increasing focus of experimental work. Here, we review recent experimental progress in areas as different as the evolution of drug resistance in cancer cells and the rewiring of transcriptional regulation circuits in vertebrates. This research reveals the importance of three major themes: multiple genetic and non-genetic mechanisms to generate phenotypic diversity, robustness in genetic systems, and adaptive landscape topography. We also discuss the mounting evidence that evolvability can evolve and the question of whether it evolves adaptively.

Isogenic populations
Populations of individuals with the same genotype.

Phenotypic plasticity
The ability of one genotype to produce more than one phenotype in response to different environmental stimuli.

Modularity
The extent to which a system can be partitioned into distinct components.

Pleiotropy
When one gene or one mutation affects multiple phenotypes.

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Evolvability research is now entering its fourth decade. Although the term was first used as early as 1932, evolvability as a scientific subdiscipline of evolutionary biology is often associated with a 1989 article by Richard Dawkins[1] describing what are now called digital organisms[2]. Today, research on evolvability is integral to multiple fields, including population genetics, quantitative genetics, molecular biology and developmental biology. Not surprisingly then, this diversity of research has led to various definitions of evolvability[3]. We here focus on one of them because we consider it the most fundamental: evolvability is the ability of a biological system to produce phenotypic variation that is both heritable and adaptive. The definition is fundamental because, first, heritable phenotypic variation is the essential raw material of evolution. Second, unless a biological system has the potential to produce variation that is adaptive (beneficial) in some environments, adaptation by natural selection is impossible. Third, the definition is broad enough to apply to fields as different as population genetics and molecular biology, which study evolvability in different ways[3].

Most early evolvability research was theoretical or guided by few experimental studies[1,3–11]. This has changed. Research on evolvability is becoming increasingly experimental and driven by advances in high-throughput technologies (BOX 1). The observations from such experiments are providing a mechanistic understanding of how living systems generate heritable adaptive variation[12]. We focus this Review on such experimental studies, which come from a diversity of fields, ranging from developmental to cancer biology. Many make no explicit mention of evolvability, yet they all shed light on the causes of evolvability and some also on its evolution. They are relevant for phenomena as different as the evolution of antibiotic resistance in bacteria and the evolutionary rescue of populations threatened by climate and other environmental change. Their insights fall into three major categories, which provide a scaffold for this Review.

The first major category encompasses molecular mechanisms that create phenotypic heterogeneity and do so not just through DNA mutations but even in the absence of such mutations. These mechanisms have become central to evolvability research because they allow isogenic populations to create phenotypic variation, some of which may facilitate survival in new or rapidly changing environments and may thus provide time for an advantageous phenotype to be reinforced or stabilized via DNA mutation, gene duplication, recombination or epigenetic modification. The second category of evidence revolves around robustness, which is central to evolvability because it allows an evolving population to explore new genotypes without detrimentally affecting essential phenotypes. The resulting genotypic diversity may serve as a springboard for subsequent mutations to generate novel phenotypes, or it may bring forth new phenotypic variation when the environment changes. The third category of evidence regards the topographical features of an adaptive landscape, such as its smoothness, and a population's location within such a landscape. These factors determine the amount of adaptive phenotypic variation that mutation can bring forth. Adaptive landscapes provide a useful geometric framework to encapsulate genotype–phenotype (or fitness) relationships that affect evolvability.

Unfortunately, space constraints prevent us from reviewing other important aspects of evolvability research, including the roles of phenotypic plasticity, organismal development, modularity and pleiotropy, as well as

Box 1 | **Methodological advances**

Our ability to study the molecular causes of evolvability has been greatly improved by recent methodological advances. For example, our growing understanding of phenotypic heterogeneity is driven by microfluidic devices and time-lapse microscopy, which provide information about the compositions, morphologies and growth rates of single cells in dynamic environments[186]. Complementary information is provided by methods such as fluorescence in situ hybridization and single-cell RNA sequencing (RNA-seq), which describe the location and abundance of mRNA transcripts, respectively[187,188]. Combined with whole-genome sequencing, such methods have detailed the molecular causes of phenotypic heterogeneity, such as how stochastic gene expression drives persistence in bacteria[26] and rare cell variability in cancer[24]. Non-single-cell methodologies have also furthered our understanding of phenotypic heterogeneity. For example, ribosome footprint profiling, which characterizes the distribution of ribosomes on mRNA transcripts[189], has detailed the prevalence of stop-codon readthrough in yeast, fly and human[39].

Several methodological advances have improved our understanding of mutational robustness and of adaptive landscapes. For example, approaches that characterize a small region of an adaptive landscape typically rely on deep mutational scanning[139], a method that combines systematic mutagenesis with high-throughput phenotypic assays. These assays include fluorescence-activated cell sorting, which can be used to measure protein functions such as fluorescence or ligand binding, as well as EMPIRIC[190], which can measure the fitness of many cells in parallel. To capture the effects of mutations in their native genomic context, genome-editing tools such as CRISPR–Cas9 can be used to introduce mutations to specific chromosomal loci[103]. Approaches that exhaustively characterize an entire (small) genotype space have profited from chip-based technologies that simultaneously assay the phenotypes of all possible genotypes[93], as well as from high-throughput in vitro selection methods that systematically enrich an initially random library of sequences for those sequences that perform a particular function, such as binding a ligand[147].

To understand how these causes of evolvability have changed over long evolutionary timescales, they are often combined with maximum likelihood methods to statistically infer and experimentally reconstruct the genotypes and phenotypes of ancient macromolecules[191].

theoretical advances. Additionally, we frame this Review primarily around mechanisms of pre-mutation evolvability and mechanisms that do not require genetic change, although we briefly discuss some mechanisms of post-mutation evolvability, in which recombination plays an especially important role[13].

Pre-mutation evolvability
Evolvability driven by new mutations.

Post-mutation evolvability
Evolvability driven by existing genetic variation within a population — for example, via recombination acting on that variation.

Gene expression noise
Variability among isogenic cells in transcript or protein abundance.

Viral latency
The ability of a virus to remain dormant in a host cell.

Competence
The ability of a cell to take up DNA from the environment.

Population bottleneck
A temporary, drastic reduction in population size.

Phenotypic heterogeneity

Heritable phenotypic variation is the raw material of natural selection, and the best-known mechanisms to create such variation are DNA mutation and recombination. However, because the role these mechanisms play in generating phenotypic variation is well established and has been extensively reviewed[13,14], we here focus on another class of mechanisms whose astonishing diversity is only beginning to come to light through recent experimental work[15]. These mechanisms create phenotypic heterogeneity without creating genetic variation.

Non-genetic mechanisms to create phenotypic heterogeneity can be found in many processes affecting the expression of genetic information. We review four such mechanisms: stochastic gene expression, errors in protein synthesis, epigenetic modifications and protein promiscuity. Each mechanism can create phenotypic variation in a population of genetically identical individuals[16]. Such variation can, for example, provide a competitive advantage to subpopulations with adaptive phenotypes in fluctuating environments[17,18]. These phenotypes may themselves be heritable, eventually made permanent by mutation or epigenetic modification, or they may simply 'buy time' for a population to adapt in other ways to an environmental challenge (FIG. 1a).

Stochastic gene expression. Stochastic gene expression, or gene expression noise, has multiple causes, including the varying efficiency of transcription and translation[19,20] as well as the regulation of gene expression by low-abundance molecules whose numbers fluctuate randomly in a cell[21] (FIG. 1b). Stochastic gene expression can create non-genetic, adaptive diversity in phenotypes as diverse as viral latency, bacterial competence, antibiotic resistance, as well as drug resistance in cancer[22–24].

One example in which stochastic gene expression causes adaptive phenotypic variation is persistence, in which some cells in an isogenic population exhibit a physiologically dormant phenotype called a persister phenotype[25]. This phenotype is adaptive because a dormant subpopulation has the potential to survive drugs that require active growth for killing, affording the persistent subpopulation time to acquire resistance-conferring DNA mutations. This phenomenon was recently demonstrated in a laboratory evolution experiment of *Escherichia coli* populations subjected to intermittent exposures of ampicillin[26], in which persistence served as a stopgap until some individuals acquired resistance-causing mutations.

Persistence arises in only a small fraction of a population; therefore, one might think that the resulting population bottleneck would hinder evolvability by reducing the supply of beneficial mutations. However, a recent study of non-small-cell lung cancer indicates that this need not be the case[27]. These cells stochastically express a persistent phenotype, mediated by an altered chromatin state[28]. A population derived from one of these cells was exposed to the drug erlotinib, which resulted in the formation of multiple persistent subpopulations. Seventeen of these subpopulations were later expanded in isolation from each other until drug resistance emerged through DNA mutations. Genetic analysis of the resistant clones uncovered several distinct resistance mechanisms, indicating that several evolutionary paths to resistance remained despite the population bottleneck. In sum, persistence can facilitate evolvability because it allows some individuals (individual cells in this example) to survive long enough to experience adaptive genetic change.

Rare cell variability is similar to persistence in that a subpopulation of cells stochastically expresses a phenotype that facilitates the evasion of drug treatment[28,29]. Rare cell variability is different from persistence in that the subpopulation is not dormant but rather exhibits a transient transcriptional state that may include the expression of resistance-conferring genes. For example, in a study of resistance evolution to the drug vemurafenib in human melanoma, rare cells transiently expressed several such genes before drug exposure, making them 'pre-resistant'[24]. After 4 weeks of drug exposure, stably resistant colonies emerged that expressed these genes at uniformly high levels and in a semi-coordinated fashion. Of 1,456 genes known to contribute to resistance, pre-resistant cells expressed 72. After 4 weeks of drug exposure, this number rose to 966. These changes were not caused by DNA mutations. Rather, drug exposure

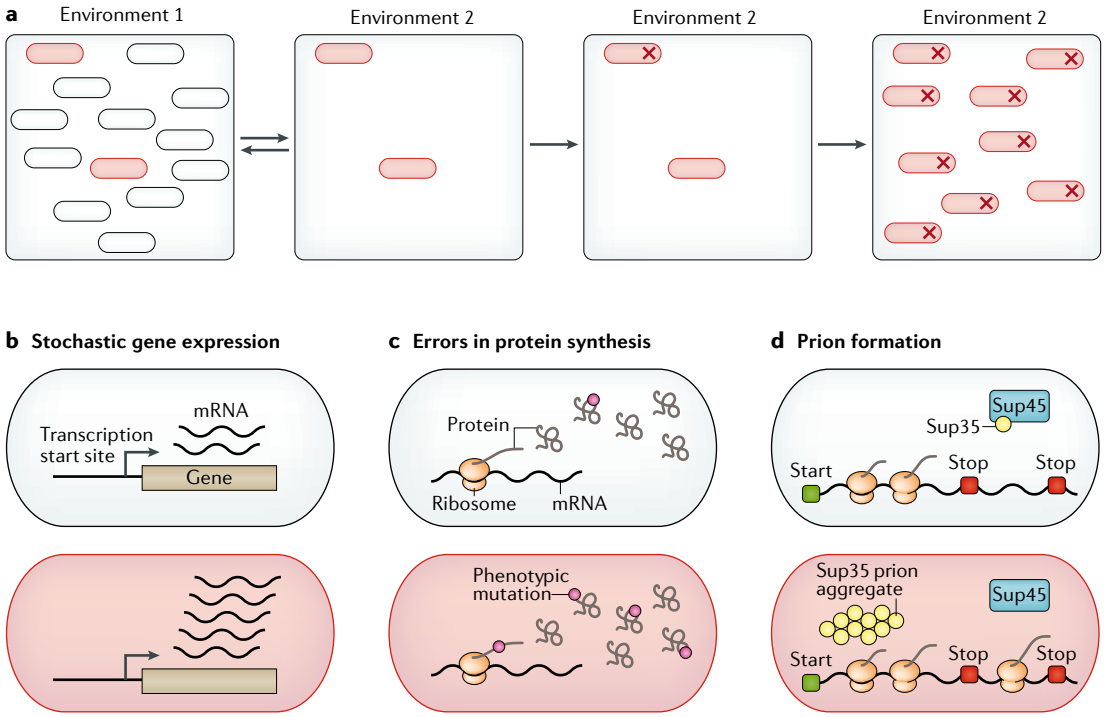

Fig. 1 | **Phenotypic heterogeneity is a cause of evolvability. a** | Phenotypic heterogeneity can generate a small subpopulation of cells that exhibits a new phenotype, such as a persister phenotype (red cells in environment 1). Such a phenotype can be adaptive because it allows a subpopulation to survive an environmental challenge, such as antibiotic exposure (environment 2). A mutation (red cross) may stabilize the phenotype, or it may generate a different phenotype that is adaptive in the new environment, such as a mutation that confers resistance to an already-tolerant bacterial cell. There are many sources of phenotypic heterogeneity. **b** | Stochastic gene expression causes mRNA transcript levels to vary among cells. **c** | Errors in protein synthesis, such as mistranslation, cause variation in the amino acid sequences of proteins that are translated from the same mRNA transcript. **d** | Epigenetic modifications, such as the yeast prion [*PSI*$^{+}$], cause variation in protein sequences, in this example via stop-codon readthrough.

initiated epigenetic cellular changes that stabilized the transiently resistant state. The transient expression of resistance-conferring genes in rare cells is not limited to melanoma but is also found in unrelated cancer cell types, suggesting that the epigenetic conversion of a rare, transient transcriptional state to a stably resistant state may often play a role in the evolvability of cancer[30]. Such stabilization of a new phenotype, even if temporary, may facilitate more permanent stabilization through genetic mutations. Examples such as these are closely related to the phenomenon of genetic assimilation, which has been studied since the 1950s[31,32].

Stochastic gene expression may also facilitate evolvability by changing how strongly mutations affect fitness, and in particular by enhancing the positive effects of beneficial mutations[33]. This was recently demonstrated using synthetic gene circuits in *Saccharomyces cerevisiae*[34], which were engineered to exhibit varying degrees of expression heterogeneity in an antifungal resistance gene. Populations harbouring a version of a circuit with high expression heterogeneity were compared with those harbouring a circuit with low expression heterogeneity. During an evolution experiment in which populations were exposed to increasing concentrations of the antifungal drug fluconazole, high-heterogeneity populations went extinct less often and evolved higher fluconazole resistance than low-heterogeneity populations. At least partly responsible were the increased beneficial effects of fluconazole resistance mutations in high-heterogeneity populations because the same resistance mutations conferred greater resistance when expressed with high expression heterogeneity than with low heterogeneity. Altering the phenotypic effects of mutations is therefore another route by which stochastic gene expression can facilitate evolvability[33].

Errors in protein synthesis. In addition to stochastic gene expression, protein synthesis errors can also create non-genetic phenotypic heterogeneity. Such errors come in many forms and occur at multiple stages of protein synthesis, including nucleotide misincorporation during transcription, tRNA misacylation during translation and kinetic trapping during protein folding[35]. Translation is particularly prone to error, with rates of mistranslation exceeding those of DNA point mutations by several orders of magnitude. Such errors are also called phenotypic mutations[36], and they include missense, readthrough and frameshift mutations. Phenotypic mutations can facilitate evolvability because they create variation in a protein pool expressed from the same gene, and some of this variation may be adaptive (FIG. 1c). For example, elevated mistranslation rates in *Mycobacterium tuberculosis* generate variation in the β-subunit of RNA polymerase, which increases resistance to the antibiotic

Genetic assimilation
A process by which a new phenotype that results from an environmental perturbation becomes genetically encoded.

Kinetic trapping
Occurs when a protein does not reach its minimum free energy structure but rather becomes trapped in a non-equilibrium structure.

rifampicin[37]. Similarly, mistranslation of CUG codons in the fungal pathogen *Candida albicans* generates variation in cell surface proteins that facilitate evasion of the host's immune system[38].

A special kind of mistranslation error is stop-codon readthrough, which is a common mechanism for generating protein variation in species as different as yeast, fly and human[39,40]. In fungi, for example, stop-codon readthrough can lead to the expression of cryptic peroxisomal signalling motifs that create variation in the cellular localization of proteins[40]. In crustacea and hexapods, DNA sequences downstream of an affected stop codon are often evolutionarily conserved, suggesting that stop-codon readthrough occurs frequently enough to affect the evolution of cryptic sequences[41,42].

Protein synthesis errors not only enhance evolvability by increasing protein diversity but also help pave the way for subsequent adaptive genetic change[43,44]. An example comes from the *S. cerevisiae* protein Idp3, an NADP-dependent isocitrate dehydrogenase that localizes to the peroxisome[45]. The Idp3 protein originated in an ancient yeast whole-genome duplication and diverged from its cytosolic ancestor Idp2 by acquiring a carboxy-terminal peroxisomal targeting signal while Idp2 remained cytosolic. Yeast species that diverged before the whole-genome duplication possess only a cytosolic *IDP2* gene, but in four of these species the gene contains a cryptic peroxisomal targeting signal in the 3′ untranslated region. This signal can be revealed via a +1 translational frameshift that bypasses the stop codon, which exposes the mistranslated protein to selection for peroxisomal targeting and function and can, for example, lead to an increase in the strength of the peroxisomal signalling motif[45]. The frameshift is induced by a sequence context that is prone to ribosomal slippage and that is also prone to single-nucleotide deletions mimicking the effect of the frameshift on protein sequence. This correlation between phenotypic and genotypic mutations thus facilitated the evolution of Idp3. Before the whole-genome duplication, Idp2 could already be expressed in two locations — in the cytosol through faithful translation and in the peroxisome through mistranslation. After the whole-genome duplication, the peroxisomal localization and function was made permanent via a single base deletion in one of the gene copies.

Epigenetic modifications. Phenotypic heterogeneity can also be caused by epigenetic changes, such as methylation of DNA and histones, alteration of chromatin structure and the changed protein conformations known as prions. For example, the prion [*PSI*$^+$] in *S. cerevisiae* is an aggregated conformation of the translational suppressor protein Sup35, which can be inherited by forming inactive complexes that convert other Sup35 proteins to the same inactive state[18]. Such aggregation reduces translational fidelity, which causes translational errors that include stop-codon readthrough events and frameshifts in other proteins[46] (FIG. 1d). Some of these errors reveal cryptic genetic variation, producing phenotypes that are heritable and that can be adaptive[18,47]. For example, [*PSI*$^+$] can improve growth on a variety of carbon and nitrogen sources, at various temperatures, and in multiple stress conditions[18,48]. The phenotypes induced by [*PSI*$^+$] and other prions can persist for generations, which provides opportunity for the phenotypes to be reinforced by mutation or recombination or to interact with existing genetic variation or new mutations to form novel, potentially adaptive phenotypes[47,49]. Recent research in this area has greatly expanded the repertoire of known prions[49–51], elucidated the mechanisms by which they confer a selective advantage[52–54] and uncovered alternative forms of protein-based inheritance[55–57]. For instance, the first bacterial prion has recently been identified[50] — the transcription terminator Rho of *Clostridium botulinum*. Rho can take on one of two conformations: a soluble form that does not affect transcription and an aggregate prion form that can self-propagate and that alters transcription, causing genome-wide transcriptomic changes. The discovery of Rho raises the exciting possibility that this cause of evolvability is ancient and predates the origin of eukaryotes.

The methylation of DNA and histones is a heritable epigenetic modification, which can create phenotypic variation that is adaptive[58,59]. A recent example comes from the study of intratumour heterogeneity in cancer[60]. Proliferative potential varies among cancer cells within the same tumour, and those cells that preserve proliferative potential can drive long-term tumour growth. Some of this variation is caused by an epigenetic modification to an enhancer that modulates the expression of the linker histone H1.0, which is involved in the compaction of chromatin. Specifically, DNA methylation of the enhancer represses the expression of the linker histone, which destabilizes nucleosome–DNA interactions, which derepresses the expression of oncogenes that support proliferative potential. Thus, variation in the epigenetic modification of a regulatory element creates variation in chromatin structure, some of which facilitates cancer cell self-renewal. This epigenetic cause of intratumour heterogeneity is found in dozens of cancers[60], and it is just one of several epigenetic causes of phenotypic heterogeneity in this disease[59].

Protein promiscuity. A fourth cause of evolvability-enhancing phenotypic heterogeneity is protein promiscuity[61,62]. Promiscuous proteins have one primary adaptive function and other secondary latent functions. Prominent examples include enzymes with 'moonlighting' catalytic activities[63,64], such as bacterial carbonic anhydrase II, which mainly catalyses the reversible hydration of carbon dioxide but also exhibits promiscuous activity towards esters[61]. Promiscuity can facilitate evolvability because it provides a reservoir of potentially adaptive protein activities that can be enhanced by gene duplication when such duplications are followed by mutations that refine different activities in different duplicates. For example, in *S. cerevisiae*, two transcription factors that are products of a past gene duplication regulate the genes involved in maltose metabolism and the genes involved in palatinose metabolism[65]. These duplicates arose from a single promiscuous transcription factor that regulated the expression of both the maltose-specific and palatinose-specific genes. After gene duplication, two single-nucleotide mutations in the

Stop-codon readthrough
When translation does not terminate at a stop codon but rather continues to extend an amino acid chain.

Prions
Proteins that propagate by inducing properly folded proteins to convert into a misfolded form, often resulting in aggregation.

Cryptic genetic variation
Genetic variation that normally causes little to no phenotypic variation but that has the potential to cause phenotypic variation in new environments or new genetic backgrounds.

Enhancer
A short DNA sequence that is bound by regulatory proteins to activate the transcription of a gene, which may be located many thousands of base pairs away.

DNA binding domain of one of the duplicates altered its binding specificity such that it could no longer bind to the promoters of the maltose-specific genes. Mutations in the coding region of the other duplicate weakened its DNA binding activity; consequently, it could activate only the maltose-specific genes because only their promoters contain multiple binding sites for the protein, which compensate for the protein's reduced activity. Gene duplication thus facilitated the partitioning of the promiscuous activity of a single transcription factor among its duplicates.

Sometimes duplication may not even be needed to reinforce a promiscuous function[66,67], and this is especially true for regulatory elements. For example, the *Drosophila santomea* gene *Neprilysin 1* evolved a novel expression pattern in the fly's optic lobe via a small number of mutations to an existing enhancer[68]. Reconstruction of the enhancer's ancestral state revealed its promiscuous activity in the optic lobe, indicating that these mutations did not generate new enhancer activity de novo but rather refined one of the enhancer's existing, latent activities.

In sum, these examples show how various forms of phenotypic heterogeneity — caused by stochastic gene expression, errors in protein synthesis, epigenetic modifications and protein promiscuity — facilitate the exploration of novel phenotypes. Some of these phenotypes may be adaptive and may be made permanent by selection for genetic or epigenetic changes that reinforce the phenotype. We emphasize that many other mechanisms to regulate molecular processes exist, and given the adaptive benefits of phenotypic heterogeneity, it is likely that they will also be implicated in producing such heterogeneity.

Robustness

Robustness to DNA mutations can be viewed as a dual, converse or opposite property to non-genetic phenotypic heterogeneity. Whereas non-genetic phenotypic heterogeneity implies that phenotypic variation exists in the absence of genetic variation, robustness implies that phenotypic variation does not exist in the presence of genetic variation because a phenotype is robust to genetic change.

Many phenotypes are to some extent robust to mutations[69,70]. Examples include the structure and biological activity of macromolecules[71], the gene expression patterns of regulatory networks[72] and the ability of metabolism to synthesize biomass[73]. Such robustness can also be enhanced in various ways. For example, DNA mutations that enhance protein stability can also enhance robustness because enhanced protein stability increases the range of mutations a protein can experience while still folding into its native structure[71]. Gene duplication can also enhance robustness because it causes gene functions to become redundant and can thus increase the incidence of mutations that can be tolerated by either duplicate[74] (but see REFS[75,76]). Chaperones such as the eukaryotic heat-shock protein Hsp90 enhance robustness in organisms as diverse as fruitflies, cave fish, plants and bacteria[77–82], although such buffering may not occur in all organisms and may not affect all genetic variation[78,83].

In each of these cases, DNA mutations can cause genetic diversity without changing a phenotype. Such cryptic genetic variation can facilitate evolvability in at least three ways. First, cryptic genetic variation may be revealed as phenotypic variation (for example, via the partial loss of function of a chaperone, via the appearance of a prion or when the environment changes[18,42,47,78,81,84,85]). This revealed variation may be enriched for adaptations[42]. Second, cryptic genetic variation provides many distinct genetic backgrounds in which the effects of new mutations can manifest themselves[86,87]. This can be advantageous because the same mutation can have different phenotypic effects — neutral, beneficial or detrimental — in different genetic backgrounds, a phenomenon caused by frequent epistatic interactions (non-additive interactions) among mutations. Finally, cryptic genetic variation may give rise to new phenotypic variation via recombination.

The study of robustness has a long history in evolvability research[69,88], but recent experimental work has greatly expanded our mechanistic understanding of how robustness facilitates the generation of adaptive phenotypic variation. These advances largely result from technological progress in areas such as deep mutational scanning and ancestral protein reconstruction (BOX 1). We highlight recent examples from individual macromolecules, from interactions between macromolecules and their ligands and from entire gene regulatory networks.

The C2H2 zinc-finger is the most prominent protein domain in many metazoans but not in other eukaryotes. It occurs in C2H2 zinc-finger transcription factors, in which multiple copies of this domain are typically arranged in tandem such that each domain contacts three or more DNA bases whose identity is determined by four base-contacting amino acids in the domain's α-helix. The diversity of DNA sequences recognized by metazoan C2H2 zinc-fingers far exceeds that of other eukaryotic C2H2 zinc-fingers, and recent research implicates robustness in their expansion and diversification[89]. Specifically, in metazoans, non-base-contacting amino acids of the C2H2 zinc-finger domain form hydrogen bonds with the DNA phosphate backbone to enhance binding energy. By contrast, the binding energy of other eukaryotic C2H2 zinc-fingers depends primarily on base-contacting amino acids. This observation suggests that the non-base-contacting amino acids of metazoan C2H2 zinc-fingers confer robustness of DNA binding to mutations in base-contacting amino acids, which facilitates the diversification of DNA binding preferences.

The evolution of steroid receptor binding preferences provides another example of how robustness facilitates evolvability. Steroid receptors are transcription factors that can be classified according to their binding preference for oestrogen response elements or steroid response elements. These two response elements are six-nucleotide-long DNA sequences that differ by just two nucleotides. The ancestral steroid receptor from which all steroid receptors descended more than 450 million years ago binds to oestrogen response elements[90]. After this protein duplicated, one daughter protein retained specificity to oestrogen elements whereas the other evolved a preference for steroid response elements.

Chaperones
Proteins that assist other proteins in folding or that refold misfolded proteins.

Epistatic interactions
Non-additive interactions between alleles in their contribution to a phenotype or fitness.

Protein domain
A distinct functional and often autonomously folding unit of a protein.

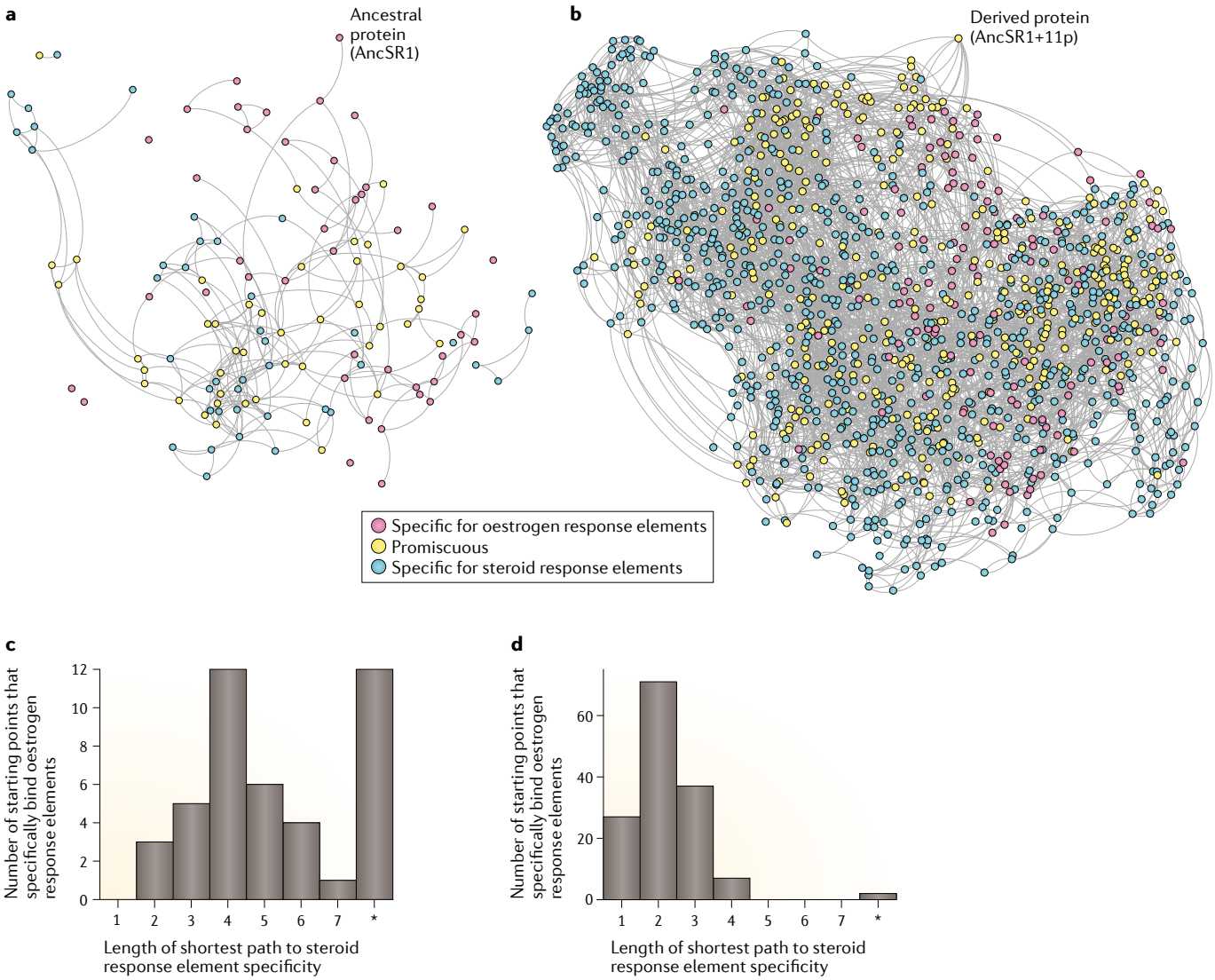

Fig. 2 | **Robustness causes evolvability by providing access to a diversity of mutational neighbourhoods. a,b** | The mutational neighbourhoods of the ancestral steroid receptor (AncSR1 in REF.[91]; part **a**) and the derived steroid receptor after 11 amino acid changes (AncSR1+11p in REF.[91]; part **b**). Each vertex (circle) corresponds to a sequence of amino acids at four sites in each protein's recognition helix: the three that historically changed binding specificity plus an adjacent site. Of all 160,000 possible such sequences in each background, only functional sequences are shown — that is, sequences that bind to the oestrogen (pink) or the steroid (blue) response elements or that promiscuously bind to both (yellow). Edges connect sequences that differ in a single amino acid. The number of functional sequences differs dramatically between the two backgrounds: 129 in the ancestral background as compared with 1,351 in the derived background. **c,d** | Moreover, the lengths of the shortest paths from a sequence that binds to the oestrogen response element to a sequence that binds to the steroid response element are much longer in the ancestral background (part **c**) than in the derived background (part **d**). The asterisk symbol indicates starting points from which there is no path to a sequence that binds to the steroid response element. Data from REF.[91].

This shift in specificity required 11 substitutions outside of the DNA binding domain and 3 substitutions within it. The 11 mutations outside of the DNA binding domain did not affect DNA binding specificity (specificity was robust to genetic changes), but they had another important consequence in that they dramatically altered the number of mutational variants capable of binding steroid response elements. Specifically, out of 160,000 possible mutational variants of the ancestral protein without the 11 mutations, only 41 specifically bound steroid response elements. By contrast, among the same 160,000 mutational variants of the ancestral protein with the 11 mutations, 829 specifically bound steroid response elements, and these variants were accessible via fewer mutations[91]. The mutational neighbourhoods of the two proteins were therefore dramatically different, and it was the robustness to mutation that facilitated access to the mutational neighbourhood that conferred higher evolvability (FIG. 2).

Not only are regulatory proteins robust to mutation, so too are the regulatory elements they target[87,92]. For example, eukaryotic transcription factors typically

bind to dozens to hundreds of distinct nucleic acid sequences[93], which tend to be mutationally interconnected, such that a mutation to a sequence that binds to a transcription factor will often generate another sequence that also binds to the transcription factor[87]. This robustness facilitates the accumulation of genetic diversity in binding sites[94], which provides distinct genetic backgrounds in which to 'test' new mutations. Some of these mutations generate binding sites for other transcription factors[87], which may lead to adaptive gene expression changes.

Gene expression patterns themselves are highly robust, not only to mutations in binding sites but also to wholesale changes in the number, identity and orientation of binding sites within regulatory regions[95] and thus to changes in the structure of gene regulatory networks[96]. Modelling work has long anticipated that such robustness can facilitate evolvability[97,98], but empirical support for this possibility was only recently provided[99]. Specifically, the highly conserved fungal transcription factor Ndt80 underwent a pronounced switch in function from an ancestral role regulating meiosis and sporulation to a derived role regulating biofilm formation. Experiments with six different extant yeast species suggest that this shift was not caused by a change in the binding specificity of Ndt80 but rather by gains and losses of binding sites for Ndt80. These changes preserved the ancestral role of Ndt80 but allowed the regulatory network controlling meiosis and sporulation to sample many architectural configurations. This sampling facilitated the discovery of a network configuration that supported the derived role of biofilm production in *C. albicans*.

In sum, these examples illustrate that robustness creates opportunities for the exploration of novel genotypes, some of which constitute or lead to new adaptations. Other pertinent examples include recent studies of robustness in viral proteins[100,101], bacterial enzymes[102], tumour suppressor genes[103], protein–protein interactions[104,105] and gene regulatory networks[106].

Adaptive landscape topography

An adaptive landscape is an analogy to a physical landscape in which each location or coordinate in a physical space corresponds to a genotype in an abstract genotype space[107] and in which the elevation at this location corresponds to the fitness of this genotype[108]. One can view adaptive evolution as a process in which populations of ever-changing genotypes explore such a landscape through random DNA mutations and recombination and in which natural selection helps such populations discover peaks or plateaus of high fitness. Adaptive landscapes are central to evolvability research because the topography of an adaptive landscape and a population's location within a landscape determine the amount of beneficial phenotypic variation that mutations can create. A smooth, single-peaked landscape facilitates evolvability because mutation can bring forth beneficial phenotypic variation from anywhere in the landscape except atop a global peak (FIG. 3a). By contrast, a rugged landscape can hinder evolvability because the local peaks it contains may attract an evolving population and preclude the generation of further beneficial phenotypic variation (FIG. 3b). Moreover, the shape of an adaptive peak — concave versus convex — affects the amount of beneficial phenotypic variation that mutation can bring forth as an evolving population ascends the peak. Until recently, most work on adaptive landscapes was theoretical, but experiments are now being increasingly used to characterize the topography of adaptive landscapes[109]. Some of these studies use organismal fitness to define the surface of a landscape[110,111] whereas others use molecular phenotypes, such as the enzymatic activity[112,113] or binding affinity[114,115] of a protein, and are therefore also referred to as genotype–phenotype landscapes[116]. The pace of this work is still accelerating, and we focus on the most recent such work.

Perhaps the most important factor affecting landscape ruggedness and the shape of adaptive peaks is epistasis — non-additive interactions between two or more mutations[117,118]. Epistasis can take different forms (FIG. 3c,d) and can occur with mutations that are individually deleterious or beneficial. For example, negative epistasis among beneficial mutations occurs when the combined effect of the mutations is smaller than the sum of the individual mutational effects[119,120] (FIG. 3c). Negative epistasis is also referred to as antagonistic or diminishing returns epistasis. Positive epistasis among beneficial mutations occurs when the combined effect of the mutations is larger than the sum of the individual mutational effects (FIG. 3c). Positive epistasis is also referred to as synergistic epistasis. The terminology used to describe epistasis can be confusing (for example, synergistic epistasis is also used to describe negative epistasis among deleterious mutations)[121], but mathematically the definition of positive and negative epistasis is straightforward. Epistasis between two mutations, A and B, can be quantified as $\varepsilon = f_{ab} + f_{AB} - f_{Ab} - f_{aB}$, where f is the phenotype or fitness of the 'wild-type', double-mutant and single-mutant genotypes, respectively. Negative epistasis occurs when $\varepsilon < 0$, whereas positive epistasis occurs when $\varepsilon > 0$.

Another important form of epistasis is sign epistasis[122]. It occurs when the sign (that is, beneficial (+) or detrimental (–)) of a double mutation differs from that of one or both of the constituent single mutations. For example, whereas both single mutations may be individually detrimental, they may be jointly beneficial. Sign epistasis creates local valleys or peaks and thus ruggedness in an adaptive landscape[118] (FIG. 3d). In doing so, it can affect the amount of adaptive variation accessible to a population, a population's evolutionary trajectory and its ability to reach a global peak. For example, global peaks may be inaccessible if all evolutionary trajectories to them require traversing one or more adaptive valleys, which is disfavoured by natural selection and possible only under restricted conditions[123,124]. With some exceptions[125–127], sign epistasis thus reduces evolvability.

A fundamental challenge in mapping an adaptive landscape is that the number of genotypes in a typical genotype space is so vast that their phenotype or fitness cannot usually be exhaustively measured. One approach to overcome this challenge uses experimental evolution

Genotype space
The space of all possible genotypes. For a nucleic acid sequence of length L, this space comprises 4^L genotypes.

Concave
A real-valued function on an interval of real numbers is concave if any line connecting two points on the graph of the function lies on or below the graph.

Convex
A real-valued function on an interval of real numbers is convex if any line connecting two points on the graph of the function lies above or on the graph.

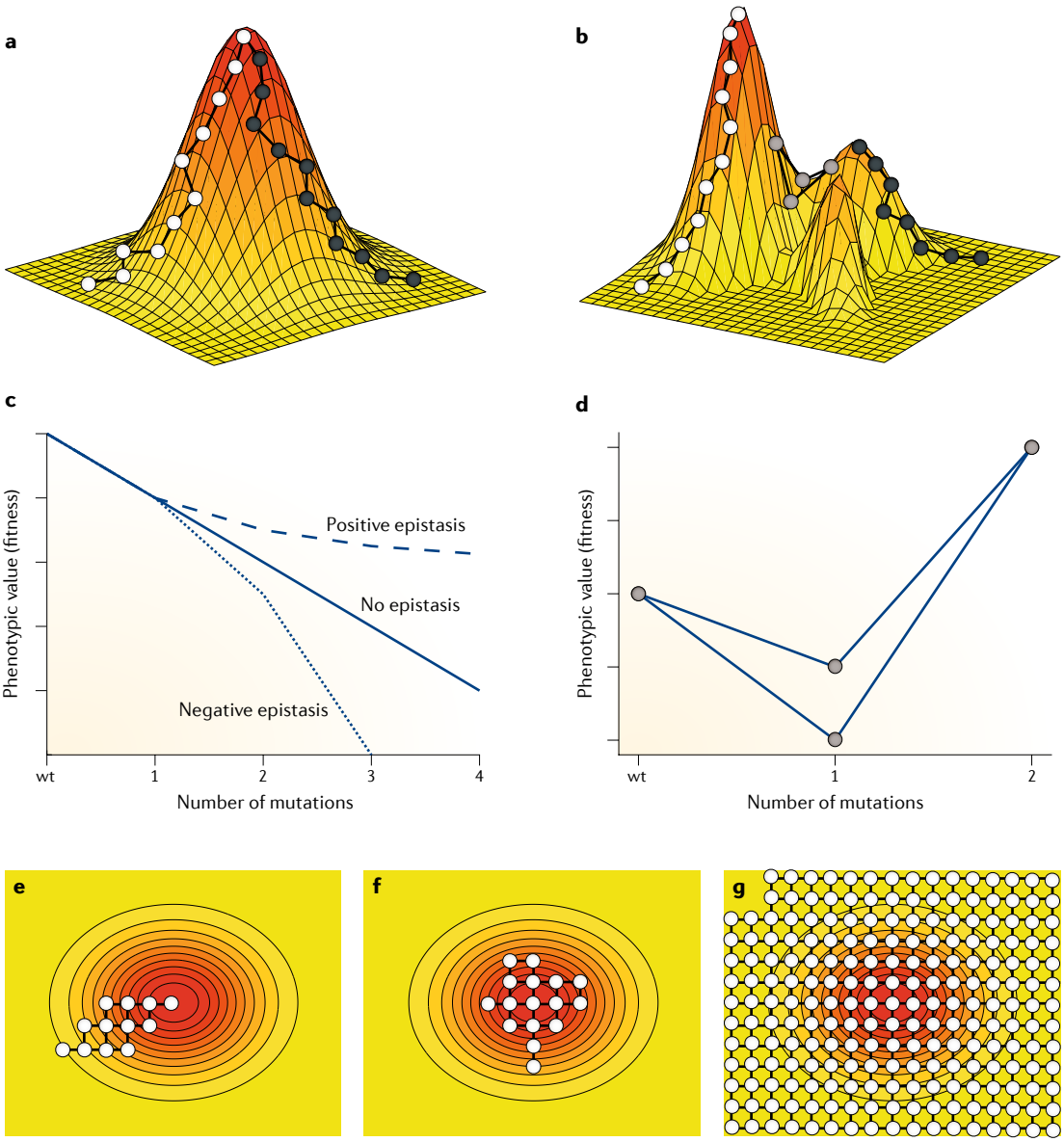

Fig. 3 | **Adaptive landscape topography influences evolvability. a** | A smooth, single-peaked landscape facilitates evolvability because mutations can create adaptive phenotypic variation from anywhere in the landscape, except atop the global peak. For example, the white and black circles denote two distinct mutational paths that start from different points in the landscape but that both converge on the global peak via a series of 'uphill' mutational steps. **b** | By contrast, a multi-peaked, or rugged landscape, hinders evolvability because an evolving population may become trapped on local, suboptimal peaks. For example, whereas the mutational path indicated by the white circles leads to the global peak, the mutational path indicated by the black circles does not. **c** | The shape of an adaptive peak is a consequence of magnitude epistasis. Specifically, positive epistasis generates peaks that are convex, whereas negative epistasis generates peaks that are concave. As a population climbs an adaptive peak, evolvability tends to increase if the peak is convex whereas it tends to decrease if the peak is concave. **d** | Landscape ruggedness is a consequence of sign epistasis, which creates adaptive valleys that may be difficult for an evolving population to cross. Grey circles correspond to those in part **b**. **e–g** | The same landscape as in part **a** but shown as 2D contour plots. Open circles indicate genotypes and edges connect genotypes that differ by a single mutation. The same landscape can be studied by systematically engineering genotypes that contain all possible combinations of a small number of mutations leading to a peak (part **e**); deep mutational scanning of a single wild-type (well-adapted) genotype, including all single mutants, many double mutants and some triple mutants (part **f**); or, in the case of small landscapes, the exhaustive enumeration of all possible genotypes (part **g**). wt, wild type.

of whole organisms[128], where the change in a population's mean fitness and genotypic composition is monitored while the population evolves for hundreds or thousands of generations in the laboratory. Such experiments show that although specific genetic changes that cause fitness increases are usually not predictable, the evolutionary trajectory of mean fitness increases can be highly predictable[129–132], suggesting that suitable statistical methods may be able to infer general statistical properties of adaptive landscape topography[133,134]. Additionally, experimental

evolution demonstrates that a population's mean fitness increase — a proxy for evolvability — depends primarily upon the fitness of the starting genotype but secondarily also upon the starting genotype itself (that is, from which location a population begins to explore an adaptive landscape)[129,135].

An important limitation of this method is that it does not allow the detailed mapping of adaptive landscape topography because evolving populations typically harbour a large number of mutations whose contributions to fitness are not easily disentangled[136,137]. Such a mapping requires more targeted approaches. One such approach is to engineer all possible genotypes in a small region of a landscape, for example, by using all combinations of the presence or absence of mutations that occurred along an adaptive evolutionary pathway or more comprehensively by using all possible combinations of mutations at a fixed number of nucleotide or amino acid sites[109] (FIG. 3e). One pertinent recent study constructed an adaptive landscape from all possible combinations of 13 amino-acid-changing mutations at 6 amino acids in the Hsp90 of *S. cerevisiae* in a high-salt environment[138]. The resulting landscape provides several fundamental insights into the evolvability of Hsp90 in this challenging environment. First, the landscape is dominated by epistasis: not a single pairwise interaction between mutations is additive. These epistatic interactions include both positive and negative epistasis, as well as sign epistasis. Second, the sign epistatic interactions produce landscape ruggedness, with five local peaks and a single global peak that conveys a 10% increase in yeast growth rate on high salt relative to the wild-type genotype. Third, although the landscape is moderately rugged, it is still highly navigable, as shown by simulated adaptive walks. These walks reveal that the global peak can be reached from nearly any starting point in the landscape. One important exception is the wild-type genotype because adaptive walks starting from this genotype tend to converge to a local peak but not to the global peak. Taken together, these observations show how epistasis can generate landscape ruggedness and that a population's location within such a rugged landscape affects the ability of mutation to bring forth heritable, adaptive phenotypic variation.

Another approach to constructing adaptive landscapes is based on deep mutational scanning[139], in which phenotypes are assayed for a large number of mutational variants of a single, typically wild-type genotype (FIG. 3f). This approach thus characterizes the immediate neighbourhood of an adaptive peak. Deep mutational scanning has been used extensively in recent years for phenotypes as different as the 'splicing-in' of an exon[116], the binding affinity[114,115] and enzymatic activity[112,113] of a protein and the fitness of an entire organism[84,110,111]. For example, a recent study employed a deep mutational scan of the wild-type sequence of the GFP from the jellyfish *Aequorea victoria* using fluorescence level to define the landscape's surface[140]. This analysis revealed a single, narrow peak centred on the wild-type sequence, with three-quarters of the single-mutant sequences displaying reduced fluorescence and half of the sequences with four mutations showing no fluorescence at all. The analysis also revealed abundant negative epistasis and very little positive epistasis. Negative epistasis produces concave peaks[141] (FIG. 3c), which reduces evolvability when a population approaches an adaptive peak because the amount of adaptive phenotypic variation accessible via mutation decreases. Conversely, positive epistasis helps create convex peaks and facilitates evolvability. These modes of epistasis also have implications for mutational robustness[141,142]. The concave peaks formed by negative epistasis confer robustness because individual mutations to genotypes on such peaks have small fitness effects. By contrast, the convex peaks formed by positive epistasis confer sensitivity to mutation, because individual mutations to genotypes on such peaks have large fitness effects. With few exceptions[143,144], a bias towards negative epistasis is among the most commonly reported features of experimentally characterized adaptive landscapes[110,111,114,115,138,140,141], in agreement with the diminishing returns epistasis regularly observed in laboratory evolution experiments[119,120,130–132].

Although deep mutational scanning and related techniques are powerful, they still render a typical genotype space sparsely sampled, and extrapolating insights from the resulting incomplete landscapes to complete landscapes is challenging[138,145,146]. Not affected by this limitation are small genotype spaces, where it is possible to assay the phenotypes of all possible genotypes[147,148] (FIG. 3g). One such genotype space is that of short transcription factor binding sites, where one can measure how strongly a transcription factor binds to thousands of different DNA sequences[93]. Such information is not just available for one but for thousands of transcription factors from multiple species[149]. Binding strength is an important molecular phenotype because it is a proxy for a factor's ability to activate or repress a target gene, and the gene expression patterns that emerge from such binding events embody fundamental biological processes, including those in development, physiology and behaviour. Importantly, the location and timing of these gene expression patterns can be fine-tuned, or altogether transformed, by mutations that affect the strength of transcription factor–DNA interactions[150,151]. The mapping of DNA sequence to binding strength can therefore be thought of as an adaptive landscape, where mutation and natural selection optimize the capacity of a DNA sequence to bind to a transcription factor.

A recent study analysed the topographies of more than 1,000 such landscapes[94]. They contain little sign epistasis and therefore typically comprise only a single peak. Similar to the landscape of yeast Hsp90 in high salinity[138], these landscapes are highly navigable. Their global peaks tend to be accessible from throughout the landscape via a series of 'uphill' mutational steps. Indeed, even at the furthest mutational distance from a global peak, more than 20% of all possible mutational paths are accessible. Such smooth landscapes facilitate evolvability because mutation can readily bring forth beneficial phenotypic variation, regardless of a population's location on the landscape.

A limitation to these approaches, as compared with experimental evolution, is that an adaptive landscape

Adaptive walks
A series of mutations that never decrease fitness.

for a single binding site or an individual gene has many fewer dimensions than an adaptive landscape for an entire genome. This limitation is important, because the valleys that separate adaptive peaks in low-dimensional landscapes may not do so in high-dimensional landscapes. The reason is that increased dimensionality may create mutational paths that bridge adaptive valleys or that transform local adaptive peaks into saddle points. Such extradimensional bypasses increase the accessibility of adaptive peaks and thus increase evolvability[5]. Long the subject of theoretical research[5,152], extradimensional bypasses have recently been uncovered in an adaptive landscape of binding affinity for the protein GB1 of streptococcal bacteria[153]. The authors analysed all 20^4 protein variants of 4 amino acid sites and sampled ~20,000 pairs of mutations that exhibited reciprocal sign epistasis (FIG. 3d). Of these pairs, ~15% exhibited an extradimensional bypass when one of the other two amino acid sites was considered. Such an increase in the mutational accessibility of adaptive peaks suggests that increasing the dimensionality of adaptive landscapes from that of individual binding sites or genes to that of entire genomes reduces landscape ruggedness and thus enhances evolvability.

The examples highlighted here are only a small sample of recent experimental studies of adaptive landscapes, with other pertinent examples in systems as different as drug delivery vehicles[154] and cancer[155]. We anticipate that the resolution and scale of such landscape studies will continue to increase as high-throughput genotyping and phenotyping technologies advance (BOX 1).

Evolvability evolving

Any cause or mechanism of evolvability could, in principle, itself be subject to evolutionary change. Three questions about such change are germane. First, can the mechanism evolve in principle; that is, is there genetic variation in it? Second, does it evolve, either in nature or in the laboratory? Third, is a change in evolvability itself adaptive or is it instead a by-product of other adaptations or of non-adaptive processes, such as developmental constraints, mutation bias or genetic drift? We discuss existing evidence pertaining to these questions for each of our three major causes of evolvability.

Evolution of phenotypic heterogeneity. Genetic mechanisms that create phenotypic heterogeneity can evolve. For example, the rate of DNA mutation is itself subject to evolutionary change[156,157] because the DNA repair enzymes that keep DNA mutations in check can themselves undergo mutations that lead to elevated mutation rates. Such evolution can be adaptive in novel environments[156,158] (for example, during colonization by *E. coli* of the mouse gut[159]). Similarly, increases in recombination rate can accelerate a population's rate of adaptation either by creating more beneficial allele combinations or by helping to eliminate deleterious mutations[160].

Non-genetic mechanisms of phenotypic heterogeneity can also evolve[161]. For example, gene expression noise levels vary genetically with promoter strength and with the strength of transcription factor binding sites[162]; stop-codon readthrough rates vary with stop-codon identity (UAG, UAA or UGA), the surrounding sequence context and the structure of mRNA[163]; the formation and activity of prions vary according to the presence of aggregation-prone amino acid sequences in prion-forming protein domains, such as glutamine/asparagine-rich sequences[164]; and protein promiscuity varies with a protein's coding sequence[61,67,105]. Thus, in each case, the factors that can affect phenotypic heterogeneity are genetically encoded and can therefore evolve.

What is more, mechanisms that create phenotypic heterogeneity do evolve, both in laboratory experiments and in nature. For example, the evolution of increased gene expression noise in *S. cerevisiae* has been reported for antifungal resistance genes in the laboratory[34] and for plasma-membrane transporters in the wild[165]. Experimental evolution of synthetic *E. coli* promoters to specific mean expression levels results in promoters with low expression noise, suggesting that the noisy expression of many natural *E. coli* promoters is an evolved property[166]. Other forms of phenotypic heterogeneity have also been successfully evolved in the laboratory, including protein promiscuity in bacteriophage-λ[67] and the stochastic switching of colony morphology in *Pseudomonas fluorescens*[17].

At least in some instances, the evolvability conferred by phenotypic heterogeneity may have evolved because it was adaptive. For example, in the experimental evolution of populations of *S. cerevisiae* exposed to antifungal stress, increased expression noise evolved in the synthetic regulatory circuits controlling an antifungal resistance gene because it enhanced the adaptive value of beneficial mutations[34]. Similarly, in the experimental evolution of populations of *P. fluorescens* exposed to environmental fluctuations, the stochastic switching of colony morphology evolved as an adaptive bet-hedging strategy[17]. Such a strategy was also observed in the experimental evolution of *E. coli* under antibiotic stress, where the stochastic expression of persister cells evolved to facilitate survival in high concentrations of antibiotic[26]. In other instances, evolvability is a by-product of other adaptations. For example, promiscuity in the host-recognition protein of bacteriophage-λ evolved as a by-product of selection for increased adsorption to the virus' native cell surface receptor[67]. Specifically, the same mutations that increased adsorption also destabilized the protein, producing λ-particles that were proficient at targeting different receptors.

Evolution of robustness. Variation in mutational robustness is found at all scales of biological organization, including the structures of macromolecules[71,147], interactions between macromolecules and their ligands[87,92], and the gene expression patterns of regulatory circuits[167]. Mutational robustness can therefore evolve; moreover, it can evolve by various means (for example, via increased protein stability[71] or via gene duplication[74]).

Mutational robustness also has evolved both in nature and in the laboratory. For example, the structures of eukaryotic microRNA precursor stem-loops are more robust to mutation than random RNA sequences with similar stem-loop structures[168], and the mutational robustness of a protein's tertiary structure tends to

Saddle points
Points on a landscape that have zero slope in at least two orthogonal directions yet are not local peaks.

Extradimensional bypasses
Accessible mutational paths to an adaptive peak that are facilitated by increasing the dimensionality of an adaptive landscape.

increase with the protein's age[169]. Directed protein evolution has demonstrated that mutational robustness of cytochrome P450 proteins can increase in sufficiently large populations[170], and experimental evolution of *S. cerevisiae* has demonstrated that gene duplications can confer mutational robustness[74].

We are not aware of experimental evidence that mutational robustness has evolved because it causes evolvability. By contrast, there is evidence that mutational robustness has evolved because it is itself adaptive[171] (for example, in viral populations exposed to chemical mutagens) because robustness provides a competitive advantage when the mutation rate is elevated[172]. In addition, mutational robustness may often evolve as a by-product of other adaptations. For example, chaperones help maintain proteome integrity during environmental stress and may buffer mutations only as a side effect. Similarly, the mutational robustness of eukaryotic microRNA precursor stem-loops is likely to be a by-product of selection for robustness of these RNA structures to temperature fluctuations[173].

Evolution of adaptive landscape topography. This cause of evolvability can also evolve: the location of an individual or a population on an adaptive landscape can change through DNA mutations or recombination, and because local landscape topography may differ in different locations, so may evolvability[91,135,138,141,147,174–176]. A comparison of the fitness effects of mutations to three orthologous TIM barrel proteins provides an illustrative example[175]. These proteins are distantly related, retaining only ~30–40% sequence identity, but they have the same fold and function. They therefore occupy different locations on the same adaptive landscape. These locations differ in their evolvability because the same mutations have different, albeit correlated, fitness effects in the three sequence backgrounds (locations). Another example is provided by the experimental evolution of two divergent yeast strains in the same laboratory conditions[129]. These strains, which differ at ~50,000 single-nucleotide sites and therefore occupy different locations on their adaptive landscape, also differ in the rate at which they adapt evolutionarily[129,177]. Analysis of quantitative trait loci partly attributes this difference in evolvability to a small subset of mutations, such as those involved in the ribosome biogenesis pathway.

The evolvability conferred by a landscape's local topography has also evolved. As shown in FIG. 2, for example, 11 substitutions occurred during the evolution of an ancient steroid hormone receptor, and this change in adaptive landscape location dramatically altered the spectrum of DNA binding phenotypes accessible via mutation[91]. An additional example comes from Lenski's long-term (>60,000 generations) evolution experiment with *E. coli* populations[178]. Here, 1 of 12 populations evolved the ability to utilize citrate and did so after 31,500 generations. The mutation needed to evolve citrate utilization conferred a fitness benefit even in the original ancestor of the experiment, but other mutations that occurred during the initial stages of the experiment conferred larger fitness benefits and created a genetic background in which the initial citrate utilization mutation no longer conferred a fitness benefit. Thus, evolution drove the population to a location on the adaptive landscape that precluded the evolution of citrate utilization. Only later did subsequent mutations bring the population to a location where this mutation was again adaptive.

The same experiment also provides further evidence for evolving evolvability[177]. Within the first 500 generations of this experiment, multiple genetically distinct subpopulations had evolved within a single population, meaning that the population had diversified from the location of the ancestral genotype to multiple new locations on the adaptive landscape. One of these subpopulations would eventually outcompete the others, but it was not the subpopulation with the highest fitness. Rather, it was a subpopulation located in a region of the adaptive landscape that had higher evolvability, as shown by 'replay experiments', in which ten replicate populations were evolved from distinct founding subpopulations (that is, from distinct locations on the adaptive landscape). The subpopulation that would eventually outcompete the others generated more beneficial phenotypic variation than the other subpopulations (that is, it had higher evolvability). After ~900 generations of evolution from these distinct landscape locations, the subpopulations evolved from the high-evolvability location tended to outcompete those evolved from other locations.

We are not aware of experimental evidence that a population's location on an adaptive landscape has evolved because it conferred evolvability. For instance, in the preceding example, evolvability evolved as a by-product of the fixation of neutral or beneficial mutations that just happened to drive one of the subpopulations towards a high-evolvability region of the landscape[177]. Non-adaptive forces may also explain the evolution of a population's location on an adaptive landscape. For example, the 11 substitutions that occurred during the evolution of an ancient steroid hormone receptor did not alter the protein's binding specificity, which suggests that genetic drift caused this change in landscape location and the corresponding dramatic shift in evolvability[90]. An alternative possibility is that this change in landscape location was caused by selection for protein functions unrelated to binding specificity.

Taken together, these examples show that the three causes of evolvability highlighted here — phenotypic heterogeneity, robustness and adaptive landscapes — are themselves subject to evolutionary change. Whether they often evolve because they confer evolvability remains a particularly challenging open question.

Outlook

Driven by technological advances, research into all three causes of evolvability is progressing in leaps and bounds. We anticipate that this progress is going to continue unabated. For example, the currently well-studied mechanisms to create the non-genetic phenotypic heterogeneity that we discuss may well be only a small subset of all pertinent mechanisms. Future work may reveal others to be important as well, such as RNA editing[179] and protein allostery[180]. In addition, we know little about how

Quantitative trait loci
Loci that explain part of the genetic basis of variation in a phenotype.

Box 2 | **Conflicts between different levels of selection**

Biological systems are hierarchically organized, with macromolecules embedded in cells, cells in whole organisms and organisms in populations. A genetic change that is beneficial on one level of this hierarchy may be detrimental on another. For example, because most random DNA mutations have detrimental effects on individuals or their offspring[192], DNA mutations that increase the DNA mutation rate itself will also be detrimental for most individuals. By contrast, DNA mutations may be advantageous for a population as a whole, especially in a stressful environment, where a few beneficial mutant individuals may ensure survival[158,193] or accelerate adaptation[156]. Such conflicts are also relevant for the evolvability mechanisms we discuss, such as those that generate non-genetic heterogeneity, because in most environments such heterogeneity will not benefit all individuals[15,22,25]. Various approaches help predict how evolution can resolve such conflicts[194–198]. Among them are multi-level selection theory[197] and kin selection theory[196]. The latter shows that higher, population-level adaptations can evolve and persist whenever populations consist of genetically highly related individuals, because in this case the genetic 'interests' of individuals are aligned with those of the population. It is relevant here that many known cases of adaptive non-genetic heterogeneity are found in clonal populations of genetically identical individuals[15], where an individual's interests are served as long as some of its clone-mates survive. Although theoretical work shows that evolvability mediated by prions such as [*PSI*$^+$] may persist in non-clonal populations of the yeast *Saccharomyces cerevisae*[85,199], extending such insights to other mechanisms of phenotypic heterogeneity, particularly non-heritable mechanisms and to a broader range of organisms, remains an important task for future work.

With respect to robustness, the dual property to phenotypic heterogeneity, we note that it is often advantageous to an individual (for example, when a mutation creates a thermodynamically more stable protein that is less prone to misfolding or inactivation[170]). Wherever this is the case, the individual-level advantage and the population-level advantage of evolvability are aligned. This makes robustness a cause of evolvability whose evolutionary origin need not involve conflict and is thus especially easy to explain. At the same time, this absence of conflict also means that it is more difficult to disentangle whether the robustness of any one trait originated in an individual-level advantage, such as the robustness that chaperones provide to proteomes[200], or in a 'second-order' advantage of evolvability, which chaperones also provide[82].

conflicts of selection may influence the evolution of such mechanisms, especially in organisms that are not clonally related (BOX 2). As for robustness, we understand its causes well for some systems such as proteins or duplicate genes, but much less well for systems of greater complexity, such as gene regulatory circuits and metabolism. The evolutionary consequences of robustness become amply clear from detailed reconstructions of the evolution of molecules such as steroid hormone receptors[91], but to date few such reconstructions are available. In the context of adaptive landscapes, we are only beginning to understand how landscape topography depends on higher-order epistasis[181,182]. Moreover, although we know that the environment can affect adaptive landscape topography, we know little about how it does[86,183]. We are also only beginning to understand how our knowledge of landscape topography may facilitate the prediction of evolutionary trajectories[109,184] or the deliberate redirection of evolving populations of pathogens towards low-evolvability regions of a landscape[185].

The three major causes of evolvability interact, but we do not fully understand how or to what effect. For example, phenotypic heterogeneity can smoothen an adaptive landscape if a genotype's overall fitness is equal to the average fitness of each of the phenotypes it brings forth[33]. Similarly, a DNA mutation that renders a protein's phenotype robust to further mutations can be viewed as displacing the genotype to a smooth region of an adaptive landscape, where further mutations have smaller phenotypic effects. However, the degree of such 'smoothing' has not been explicitly characterized for any experimentally studied landscape. When an organism generates non-genetic adaptive variation in phenotypes, it creates two or more phenotypes from the same genotype, but any one adaptive phenotype can be stabilized by DNA mutations only if the starting genotype resides in a region of an adaptive landscape where some of its mutants provide such stabilization. We do not know the extent to which non-genetic mechanisms that create phenotypic variation and increase evolvability ensure that the variation they cause can be genetically stabilized. Finally, because a phenotype's robustness to genetic and to non-genetic change are often correlated[69], genotypes that are especially robust to DNA mutations may also bring forth less phenotypic heterogeneity by non-genetic means. If so, trade-offs between robustness and non-genetic mechanisms to create phenotypic heterogeneity may exist, and these trade-offs are well worth exploring.

A final frontier regards the evolution of the various causes of evolvability. As we have shown, there is ample evidence that all three causes are subject to evolutionary change. However, we have less information about whether their existence reflects an adaptive value of evolvability. Does increased mutational robustness at least sometimes come about because it enhances evolvability? Has the ruggedness of some adaptive landscapes decreased in the course of evolution, and, if so, is it because reduced ruggedness increases evolvability? Questions such as these are fascinating and profound because an affirmative answer means that life itself can help create the conditions that ensure its advancement.

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1. Dawkins, R. in *Artificial Life: The Proceedings of an Interdisciplinary Workshop on the Synthesis and Simulation of Living Systems* (ed. Langton, C. G.) 201–220 (Addison-Wesley, Reading, MA, 1989). **This influential discussion of evolvability and its evolution is set in the context of artificial embryological systems.**
2. Nuno de la Rosa, L. Computing the extended synthesis: mapping the dynamics and conceptual structure of the evolvability research front. *J. Exp. Zool. B Mol. Dev. Evol.* **328**, 395–411 (2017).
3. Pigliucci, M. Is evolvability evolvable? *Nat. Rev. Genet.* **9**, 75–82 (2008).
4. Alberch, P. From genes to phenotype: dynamical systems and evolvability. *Genetica* **84**, 5–11 (1991).
5. Conrad, M. The geometry of evolution. *Biosystems* **24**, 61–81 (1990).
6. Hansen, T. F. Is modularity necessary for evolvability? Remarks on the relationship between pleiotropy and evolvability. *Biosystems* **69**, 83–94 (2003).
7. Houle, D. Comparing evolvability and variability of quantitative traits. *Genetics* **130**, 195–204 (1992).
8. Kauffman, S. A. Requirements for evolvability in complex systems — orderly dynamics and frozen components. *Phys. D* **42**, 135–152 (1990).
9. Kirschner, M. & Gerhart, J. Evolvability. *Proc. Natl Acad. Sci. USA* **95**, 8420–8427 (1998).
10. Wagner, A. Does evolutionary plasticity evolve? *Evolution* **50**, 1008–1023 (1996).
11. Wagner, G. P. & Altenberg, L. Perspective: complex adaptations and the evolution of evolvability. *Evolution* **50**, 967–976 (1996).
12. Diaz Arenas, C. & Cooper, T. F. Mechanisms and selection of evolvability: experimental evidence. *FEMS Microbiol. Rev.* **37**, 572–582 (2013).
13. Masel, J. & Trotter, M. V. Robustness and evolvability. *Trends Genet.* **26**, 406–414 (2010).
14. Galhardo, R. S., Hastings, P. J. & Rosenberg, S. M. Mutation as a stress response and the regulation of evolvability. *Crit. Rev. Biochem. Mol. Biol.* **42**, 399–435 (2007).
15. Ackermann, M. A functional perspective on phenotypic heterogeneity in microorganisms. *Nat. Rev. Microbiol.* **13**, 497–508 (2015).

16. Tawfik, D. S. Messy biology and the origins of evolutionary innovations. *Nat. Chem. Biol.* **6**, 692–696 (2010).
17. Beaumont, H. J., Gallie, J., Kost, C., Ferguson, G. C. & Rainey, P. B. Experimental evolution of bet hedging. *Nature* **462**, 90–93 (2009).
18. True, H. L. & Lindquist, S. L. A yeast prion provides a mechanism for genetic variation and phenotypic diversity. *Nature* **407**, 477–483 (2000).
19. Blake, W. J., M., K. A., Cantor, C. R. & Collins, J. J. Noise in eukaryotic gene expression. *Nature* **422**, 633–637 (2003).
20. Ozbudak, E. M., Thattai, M., Kurtser, I., Grossman, A. D. & van Oudenaarden, A. Regulation of noise in the expression of a single gene. *Nat. Genet.* **31**, 69–73 (2002).
21. Elowitz, M. B., Levine, A. J., Siggia, E. D. & Swain, P. S. Stochastic gene expression in a single cell. *Science* **297**, 1183–1186 (2002).
22. Raj, A. & van Oudenaarden, A. Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell* **135**, 216–226 (2008).
23. Sanchez-Romero, M. A. & Casadesus, J. Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. *Proc. Natl Acad. Sci. USA* **111**, 355–360 (2014).
24. Shaffer, S. M. et al. Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. *Nature* **546**, 431–435 (2017).
25. Harms, A., Maisonneuve, E. & Gerdes, K. Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science* **354**, aaf4268 (2016).
26. Levin-Reisman, I. et al. Antibiotic tolerance facilitates the evolution of resistance. *Science* **355**, 826–830 (2017).
This study shows that tolerance against ampicillin facilitates the evolution of resistance.
27. Ramirez, M. et al. Diverse drug-resistance mechanisms can emerge from drug-tolerant cancer persister cells. *Nat. Commun.* **7**, 10690 (2016).
28. Sharma, S. V. et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* **141**, 69–80 (2010).
29. Spencer, S. L., Gaudet, S., Albeck, J. G., Burke, J. M. & Sorger, P. K. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. *Nature* **459**, 428–432 (2009).
30. Frank, S. A. & Rosner, M. R. Nonheritable cellular variability accelerates the evolutionary processes of cancer. *PLOS Biol.* **10**, e1001296 (2012).
31. West-Eberhard, M. *Developmental plasticity and evolution* (Oxford Univ. Press, 2003).
32. Waddington, C. H. The genetic assimilation of an acquired character. *Evolution* **7**, 118–126 (1953).
33. Frank, S. A. Natural selection. II. Developmental variability and evolutionary rate. *J. Evol. Biol.* **24**, 2310–2320 (2011).
34. Bodi, Z. et al. Phenotypic heterogeneity promotes adaptive evolution. *PLOS Biol.* **15**, e2000644 (2017).
35. Drummond, D. A. & Wilke, C. O. The evolutionary consequences of erroneous protein synthesis. *Nat. Rev. Genet.* **10**, 715–724 (2009).
36. Goldsmith, M. & Tawfik, D. S. Potential role of phenotypic mutations in the evolution of protein expression and stability. *Proc. Natl Acad. Sci. USA* **106**, 6197–6202 (2009).
37. Javid, B. et al. Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. *Proc. Natl Acad. Sci. USA* **111**, 1132–1137 (2014).
38. Miranda, I. et al. *Candida albicans* CUG mistranslation is a mechanism to create cell surface variation. *mBio* **4**, e00285-13 (2013).
39. Dunn, J. G., Foo, C. K., Belletier, N. G., Gavis, E. R. & Weissman, J. S. Ribosome profiling reveals pervasive and regulated stop codon readthrough in *Drosophila melanogaster*. *eLife* **2**, e01179 (2013).
40. Freitag, J., Ast, J. & Bolker, M. Cryptic peroxisomal targeting via alternative splicing and stop codon read-through in fungi. *Nature* **485**, 522–525 (2012).
41. Jungreis, I. et al. Evolutionary dynamics of abundant stop codon readthrough. *Mol. Biol. Evol.* **33**, 3108–3132 (2016).
42. Masel, J. Cryptic genetic variation is enriched for potential adaptations. *Genetics* **172**, 1985–1991 (2006).
43. Whitehead, D. J., Wilke, C. O., Vernazobres, D. & Bornberg-Bauer, E. The look-ahead effect of phenotypic mutations. *Biol. Direct* **3**, 18 (2008).
44. Borenstein, E., Meilijson, I. & Ruppin, E. The effect of phenotypic plasticity on evolution in multipeaked fitness landscapes. *J. Evol. Biol.* **19**, 1555–1570 (2006).
45. Yanagida, H. et al. The evolutionary potential of phenotypic mutations. *PLOS Genet.* **11**, e1005445 (2015).
This study demonstrates that in the natural history of *S. cerevisiae*, a phenotypic mutation was reinforced via gene duplication and mutation.
46. Baudin-Baillieu, A. et al. Genome-wide translational changes induced by the prion [*PSI*$^+$]. *Cell Rep.* **8**, 439–448 (2014).
47. True, H. L., Berlin, I. & Lindquist, S. L. Epigenetic regulation of translation reveals hidden genetic variation to produce complex traits. *Nature* **431**, 184–187 (2004).
48. Tyedmers, J., Madariaga, M. L. & Lindquist, S. Prion switching in response to environmental stress. *PLOS Biol.* **6**, e294 (2008).
49. Halfmann, R. et al. Prions are a common mechanism for phenotypic inheritance in wild yeasts. *Nature* **482**, 363–368 (2012).
50. Yuan, A. H. & Hochschild, A. A bacterial global regulator forms a prion. *Science* **355**, 198–201 (2017).
This article discusses the discovery of the first prokaryotic prion, the transcription terminator Rho of *C. botulinum*.
51. Chakrabortee, S. et al. Luminidependens (LD) is an *Arabidopsis* protein with prion behavior. *Proc. Natl Acad. Sci. USA* **113**, 6065–6070 (2016).
52. Jarosz, D. F. et al. Cross-kingdom chemical communication drives a heritable, mutually beneficial prion-based transformation of metabolism. *Cell* **158**, 1083–1093 (2014).
53. Newby, G. A. & Lindquist, S. Pioneer cells established by the [*SWI*$^+$] prion can promote dispersal and out-crossing in yeast. *PLOS Biol.* **15**, e2003476 (2017).
This paper presents a lucid description of the mechanism by which the yeast prion [*SWI*$^+$] confers a selective advantage. [*SWI*$^+$] enhances cells' ability to disperse in water and increases the likelihood of mating with dissimilar partners, thus facilitating migration, the colonization of new habitats and genetic diversification.
54. Suzuki, G., Shimazu, N. & Tanaka, M. A yeast prion, Mod5, promotes acquired drug resistance and cell survival under environmental stress. *Science* **336**, 355–359 (2012).
55. Chakrabortee, S. et al. Intrinsically disordered proteins drive emergence and inheritance of biological traits. *Cell* **167**, 369–381.e12 (2016).
56. Franzmann, T. M. et al. Phase separation of a yeast prion protein promotes cellular fitness. *Science* **359**, eaao5654 (2018).
57. Riback, J. A. et al. Stress-triggered phase separation is an adaptive, evolutionarily tuned response. *Cell* **168**, 1028–1040 (2017).
58. Avery, S. V. Microbial cell individuality and the underlying sources of heterogeneity. *Nat. Rev. Microbiol.* **4**, 577–587 (2006).
59. Flavahan, W. A., Gaskell, E. & Bernstein, B. E. Epigenetic plasticity and the hallmarks of cancer. *Science* **357**, eaal2380 (2017).
60. Torres, C. M. et al. The linker histone H1.0 generates epigenetic and functional intratumor heterogeneity. *Science* **353**, aaf1644 (2016).
This study provides a mechanistic account of how an epigenetic modification to a regulatory element creates tumour-maintaining cells.
61. Aharoni, A. et al. The 'evolvability' of promiscuous protein functions. *Nat. Genet.* **37**, 73–76 (2005).
62. Tokuriki, N. & Tawfik, D. S. Protein dynamism and evolvability. *Science* **324**, 203–207 (2009).
63. Copley, S. D. Enzymes with extra talents: moonlighting functions and catalytic promiscuity. *Curr. Opin. Chem. Biol.* **7**, 265–272 (2003).
64. Notebaart, R. A. et al. Network-level architecture and the evolutionary potential of underground metabolism. *Proc. Natl Acad. Sci. USA* **111**, 11762–11767 (2014).
65. Pougach, K. et al. Duplication of a promiscuous transcription factor drives the emergence of a new regulatory network. *Nat. Commun.* **5**, 4868 (2014).
66. Sayou, C. et al. A promiscuous intermediate underlies the evolution of LEAFY DNA binding specificity. *Science* **343**, 645–648 (2014).
67. Petrie, K. L. et al. Destabilizing mutations encode nongenetic variation that drives evolutionary innovation. *Science* **359**, 1542–1545 (2018).
68. Rebeiz, M., Jikomes, N., Kassner, V. A. & Carroll, S. B. Evolutionary origin of a novel gene expression pattern through co-option of the latent activities of existing regulatory sequences. *Proc. Natl Acad. Sci. USA* **108**, 10036–10043 (2011).
69. Wagner, A. *Robustness and evolvability in living systems* (Princeton Univ. Press, 2005).
70. Fares, M. A. The origins of mutational robustness. *Trends Genet.* **31**, 373–381 (2015).
71. Bloom, J. D., Labthavikul, S. T., Otey, C. R. & Arnold, F. H. Protein stability promotes evolvability. *Proc. Natl Acad. Sci. USA* **103**, 5869–5874 (2006).
72. Ingolia, N. T. Topology and robustness in the *Drosophila* segment polarity network. *PLOS Biol.* **2**, e123 (2004).
73. Segre, D., Vitkup, D. & Church, G. Analysis of optimality in natural and perturbed metabolic networks. *Proc. Natl Acad. Sci. USA* **99**, 15112–15117 (2002).
74. Keane, O. M., Toft, C., Carretero-Paulet, L., Jones, G. W. & Fares, M. A. Preservation of genetic and regulatory robustness in ancient gene duplicates of *Saccharomyces cerevisiae*. *Genome Res.* **24**, 1830–1841 (2014).
75. Diss, G. et al. Gene duplication can impart fragility, not robustness, in the yeast protein interaction network. *Science* **355**, 630–634 (2017).
76. Baker, C. R., Hanson-Smith, V. & Johnson, A. D. Following gene duplication, paralog interference constrains transcriptional circuit evolution. *Science* **342**, 104–108 (2013).
77. Aguilar-Rodriguez, J. et al. The molecular chaperone DnaK is a source of mutational robustness. *Genome Biol. Evol.* **8**, 2979–2991 (2016).
78. Jarosz, D. F. & Lindquist, S. Hsp90 and environmental stress transform the adaptive value of natural genetic variation. *Science* **330**, 1820–1824 (2010).
79. Queitsch, C., Sangster, T. A. & Lindquist, S. Hsp90 as a capacitor of phenotypic variation. *Nature* **417**, 618–624 (2002).
80. Rohner, N. et al. Cryptic variation in morphological evolution: Hsp90 as a capacitor for loss of eyes in cavefish. *Science* **342**, 1372–1375 (2013).
81. Rutherford, S. L. & Lindquist, S. Hsp90 as a capacitor for morphological evolution. *Nature* **396**, 336–342 (1998).
82. Tokuriki, N. & Tawfik, D. S. Chaperonin overexpression promotes genetic variation and enzyme evolution. *Nature* **459**, 668–673 (2009).
83. Geiler-Samerotte, K. A., Zhu, Y. O., Goulet, B. E., Hall, D. W. & Siegal, M. L. Selection transforms the landscape of genetic variation interacting with Hsp90. *PLOS Biol.* **14**, e2000465 (2016).
84. Stiffler, M. A., Hekstra, D. R. & Ranganathan, R. Evolvability as a function of purifying selection in TEM-1 β-lactamase. *Cell* **160**, 882–892 (2015).
This study shows that the synergism between mutational robustness and evolvability in Tem1 β-lactamase depends upon the strength of purifying selection for ampicillin resistance.
85. Masel, J. & Bergman, A. The evolution of the evolvability properties of the yeast prion [*PSI*$^+$]. *Evolution* **57**, 1498–1512 (2003).
86. Hayden, E. J., Ferrada, E. & Wagner, A. Cryptic genetic variation promotes rapid evolutionary adaptation in an RNA enzyme. *Nature* **474**, 92–95 (2011).
87. Payne, J. L. & Wagner, A. The robustness and evolvability of transcription factor binding sites. *Science* **343**, 875–877 (2014).
88. de Visser, J. A. et al. Perspective: evolution and detection of genetic robustness. *Evolution* **57**, 1959–1972 (2003).
89. Najafabadi, H. S. et al. Non-base-contacting residues enable kaleidoscopic evolution of metazoan C2H2 zinc finger DNA binding. *Genome Biol.* **18**, 167 (2017).
This article provides a mechanistic explanation of the expansion and diversification of metazoan C2H2 zinc-finger transcription factors.
90. McKeown, A. N. et al. Evolution of DNA specificity in a transcription factor family produced a new gene regulatory module. *Cell* **159**, 58–68 (2014).
91. Starr, T. N., Picton, L. K. & Thornton, J. W. Alternative evolutionary histories in the sequence space of an ancient protein. *Nature* **549**, 409–413 (2017).
92. Payne, J. L., Khalid, F. & Wagner, A. RNA-mediated gene regulation is less evolvable than transcriptional regulation. *Proc. Natl Acad. Sci. USA* **115**, E3481–E3490 (2018).
93. Berger, M. F. et al. Compact, universal DNA microarrays to comprehensively determine transcription-factor binding site specificities. *Nat. Biotechnol.* **24**, 1429–1435 (2006).
94. Aguilar-Rodriguez, J., Payne, J. L. & Wagner, A. A thousand empirical adaptive landscapes and their navigability. *Nat. Ecol. Evol.* **1**, 45 (2017).

95. Weirauch, M. T. & Hughes, T. R. Conserved expression without conserved regulatory sequence: the more things change, the more they stay the same. *Trends Genet.* **26**, 66–74 (2010).
96. Tsong, A. E., Tuch, B. B., Li, H. & Johnson, A. D. Evolution of alternative transcriptional circuits with identical logic. *Nature* **443**, 415–420 (2006).
97. Ciliberti, S., Martin, O. C. & Wagner, A. Innovation and robustness in complex regulatory gene networks. *Proc. Natl Acad. Sci. USA* **104**, 13591–13596 (2007).
98. Martin, O. C. & Wagner, A. Effects of recombination on complex regulatory circuits. *Genetics* **183**, 673–684 (2009).
99. Nocedal, I., Mancera, E. & Johnson, A. D. Gene regulatory network plasticity predates a switch in function of a conserved transcription regulator. *eLife* **6**, e23250 (2017).
100. Doud, M. B. & Bloom, J. D. Accurate measurement of the effects of all amino-acid mutations on influenza hemagglutinin. *Viruses* **8**, E155 (2016).
101. Haddox, H. K., Dingens, A. S. & Bloom, J. D. Experimental estimation of the effects of all amino-acid mutations to HIV's envelope protein on viral replication in cell culture. *PLOS Pathog.* **12**, e1006114 (2016).
102. Jacquier, H. et al. Capturing the mutational landscape of the β-lactamase TEM-1. *Proc. Natl Acad. Sci. USA* **110**, 13067–13072 (2013).
103. Findlay, G. M., Boyle, E. A., Hause, R. J., Klein, J. C. & Shendure, J. Saturation editing of genomic regions by multiplex homology-directed repair. *Nature* **513**, 120–123 (2014).
104. Podgornaia, A. I. & Laub, M. T. Pervasive degeneracy and epistasis in a protein-protein interface. *Science* **347**, 673–677 (2015).
105. Aakre, C. D. et al. Evolving new protein-protein interaction specificity through promiscuous intermediates. *Cell* **163**, 594–606 (2015).
106. Crombach, A., Wotton, K. R., Jimenez-Guri, E. & Jaeger, J. Gap gene regulatory dynamics evolve along a genotype network. *Mol. Biol. Evol.* **33**, 1293–1307 (2016).
107. Smith, J. M. Natural selection and the concept of a protein space. *Nature* **225**, 563–564 (1970).
108. Wright, S. The roles of mutation, inbreeding, crossbreeding and selection in evolution. *Proc. Sixth Int. Congr. Genet.* **1**, 356–366 (1932).
109. de Visser, J. A. & Krug, J. Empirical fitness landscapes and the predictability of evolution. *Nat. Rev. Genet.* **15**, 480–490 (2014).
110. Li, C., Qian, W., Maclean, C. J. & Zhang, J. The fitness landscape of a tRNA gene. *Science* **352**, 837–840 (2016).
111. Puchta, O. et al. Network of epistatic interactions within a yeast snoRNA. *Science* **352**, 840–844 (2016).
References 110 and 111 are, at present, the highest-resolution characterizations of local adaptive landscape topography, in which the landscape surface represents organismal fitness.
112. Qiu, C. et al. High-resolution phenotypic landscape of the RNA polymerase II trigger loop. *PLOS Genet.* **12**, e1006321 (2016).
113. Wrenbeck, E. E., Azouz, L. R. & Whitehead, T. A. Single-mutation fitness landscapes for an enzyme on multiple substrates reveal specificity is globally encoded. *Nat. Commun.* **8**, 15695 (2017).
114. Melamed, D., Young, D. L., Gamble, C. E., Miller, C. R. & Fields, S. Deep mutational scanning of an RRM domain of the *Saccharomyces cerevisiae* poly(A)-binding protein. *RNA* **19**, 1537–1551 (2013).
115. Olson, C. A., Wu, N. C. & Sun, R. A comprehensive biophysical description of pairwise epistasis throughout an entire protein domain. *Curr. Biol.* **24**, 2643–2651 (2014).
116. Julien, P., Minana, B., Baeza-Centurion, P., Valcarcel, J. & Lehner, B. The complete local genotype-phenotype landscape for the alternative splicing of a human exon. *Nat. Commun.* **7**, 11558 (2016).
117. Poelwijk, F. J., Kiviet, D. J., Weinreich, D. M. & Tans, S. J. Empirical fitness landscapes reveal accessible evolutionary paths. *Nature* **445**, 383–386 (2007).
118. Poelwijk, F. J., Tanase-Nicola, S., Kiviet, D. J. & Tans, S. J. Reciprocal sign epistasis is a necessary condition for multi-peaked fitness landscapes. *J. Theor. Biol.* **272**, 141–144 (2011).
119. Chou, H. H., Chiu, H. C., Delaney, N. F., Segre, D. & Marx, C. J. Diminishing returns epistasis among beneficial mutations decelerates adaptation. *Science* **332**, 1190–1192 (2011).
120. Khan, A. I., Dinh, D. M., Schneider, D., Lenski, R. E. & Cooper, T. F. Negative epistasis between beneficial mutations in an evolving bacterial population. *Science* **332**, 1193–1196 (2011).
121. Elena, S. F. & Lenski, R. E. Test of synergistic interactions among deleterious mutations in bacteria. *Nature* **390**, 395–398 (1997).
122. Weinreich, D. M., Watson, R. A. & Chao, L. Perspective: Sign epistasis and genetic constraint on evolutionary trajectories. *Evolution* **59**, 1165–1174 (2005).
123. Weinreich, D. M. & Chao, L. Rapid evolutionary escape by large populations from local fitness peaks is likely in nature. *Evolution* **59**, 1175–1182 (2005).
124. Iwasa, Y., Michor, F. & Nowak, M. A. Stochastic tunnels in evolutionary dynamics. *Genetics* **166**, 1571–1579 (2004).
125. Anderson, D. W., McKeown, A. N. & Thornton, J. W. Intermolecular epistasis shaped the function and evolution of an ancient transcription factor and its DNA binding sites. *eLife* **4**, e07864 (2015).
126. Lagator, M., Sarikas, S., Acar, H., Bollback, J. P. & Guet, C. C. Regulatory network structure determines patterns of intermolecular epistasis. *eLife* **6**, e28921 (2017).
References 125 and 126 show that intermolecular sign epistasis can facilitate, rather than impede, evolvability.
127. Palmer, A. C. et al. Delayed commitment to evolutionary fate in antibiotic resistance fitness landscapes. *Nat. Commun.* **6**, 7385 (2015).
128. Buckling, A., Maclean, R. C., Brockhurst, M. A. & Colegrave, N. The Beagle in a bottle. *Nature* **457**, 824–829 (2009).
129. Jerison, E. R. et al. Genetic variation in adaptability and pleiotropy in budding yeast. *eLife* **6**, e27167 (2017).
130. Kryazhimskiy, S., Rice, D. P., Jerison, E. R. & Desai, M. M. Global epistasis makes adaptation predictable despite sequence-level stochasticity. *Science* **344**, 1519–1522 (2014).
131. Wunsche, A. et al. Diminishing-returns epistasis decreases adaptability along an evolutionary trajectory. *Nat. Ecol. Evol.* **1**, 61 (2017).
132. Wiser, M. J., Ribeck, N. & Lenski, R. E. Long-term dynamics of adaptation in asexual populations. *Science* **342**, 1364–1367 (2013).
133. Good, B. H. & Desai, M. M. The impact of macroscopic epistasis on long-term evolutionary dynamics. *Genetics* **199**, 177–190 (2015).
134. Kryazhimskiy, S., Tkacik, G. & Plotkin, J. B. The dynamics of adaptation on correlated fitness landscapes. *Proc. Natl Acad. Sci. USA* **106**, 18638–18643 (2009).
135. Gifford, D. R., Toll-Riera, M. & MacLean, R. C. Epistatic interactions between ancestral genotype and beneficial mutations shape evolvability in *Pseudomonas aeruginosa*. *Evolution* **70**, 1659–1666 (2016).
136. Good, B. H., McDonald, M. J., Barrick, J. E., Lenski, R. E. & Desai, M. M. The dynamics of molecular evolution over 60,000 generations. *Nature* **551**, 45–50 (2017).
137. Venkataram, S. et al. Development of a comprehensive genotype-to-fitness map of adaptation-driving mutations in yeast. *Cell* **166**, 1585–1596.e22 (2016).
138. Bank, C., Matuszewski, S., Hietpas, R. T. & Jensen, J. D. On the (un)predictability of a large intragenic fitness landscape. *Proc. Natl Acad. Sci. USA* **113**, 14085–14090 (2016).
139. Fowler, D. M. & Fields, S. Deep mutational scanning: a new style of protein science. *Nat. Methods* **11**, 801–807 (2014).
140. Sarkisyan, K. S. et al. Local fitness landscape of the green fluorescent protein. *Nature* **533**, 397–401 (2016).
141. Bank, C., Hietpas, R. T., Jensen, J. D. & Bolon, D. N. A systematic survey of an intragenic epistatic landscape. *Mol. Biol. Evol.* **32**, 229–238 (2015).
142. Bershtein, S., Segal, M., Bekerman, R., Tokuriki, N. & Tawfik, D. S. Robustness-epistasis link shapes the fitness landscape of a randomly drifting protein. *Nature* **444**, 929–932 (2006).
143. Trindade, S. et al. Positive epistasis drives the acquisition of multidrug resistance. *PLOS Genet.* **5**, e1000578 (2009).
144. Steinberg, B. & Ostermeier, M. Environmental changes bridge evolutionary valleys. *Sci. Adv.* **2**, e1500921 (2016).
145. du Plessis, L., Leventhal, G. E. & Bonhoeffer, S. How good are statistical models at approximating complex fitness landscapes? *Mol. Biol. Evol.* **33**, 2454–2468 (2016).
146. Otwinowski, J. & Plotkin, J. B. Inferring fitness landscapes by regression produces biased estimates of epistasis. *Proc. Natl Acad. Sci. USA* **111**, E2301–E2309 (2014).
147. Jimenez, J. I., Xulvi-Brunet, R., Campbell, G. W., Turk-MacLeod, R. & Chen, I. A. Comprehensive experimental fitness landscape and evolutionary network for small RNA. *Proc. Natl Acad. Sci. USA* **110**, 14984–14989 (2013).
148. Rowe, W. et al. Analysis of a complete DNA-protein affinity landscape. *J. R. Soc. Interface* **7**, 397–408 (2010).
149. Weirauch, M. T. et al. Determination and inference of eukaryotic transcription factor sequence specificity. *Cell* **158**, 1431–1443 (2014).
150. Sharon, E. et al. Inferring gene regulatory logic from high-throughput measurements of thousands of systematically designed promoters. *Nat. Biotechnol.* **30**, 521–530 (2012).
151. Shultzaberger, R. K., Malashock, D. S., Kirsch, J. F. & Eisen, M. B. The fitness landscapes of *cis*-acting binding sites in different promoter and environmental contexts. *PLOS Genet.* **6**, e1001042 (2010).
152. Gavrilets, S. Evolution and speciation on holey adaptive landscapes. *Trends Ecol. Evol.* **12**, 307–312 (1997).
153. Wu, N. C., Dai, L., Olson, C. A., Lloyd-Smith, J. O. & Sun, R. Adaptation in protein fitness landscapes is facilitated by indirect paths. *eLife* **5**, e16965 (2016).
154. Hartman, E. C. et al. Quantitative characterization of all single amino acid variants of a viral capsid-based drug delivery vehicle. *Nat. Commun.* **9**, 1385 (2018).
155. Rogers, Z. N. et al. Mapping the in vivo fitness landscape of lung adenocarcinoma tumor suppression in mice. *Nat. Genet.* **50**, 483–486 (2018).
156. Sniegowski, P. D., Gerrish, P. J. & Lenski, R. E. Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* **387**, 703–705 (1997).
157. Wielgoss, S. et al. Mutation rate dynamics in a bacterial population reflect tension between adaptation and genetic load. *Proc. Natl Acad. Sci. USA* **110**, 222–227 (2013).
158. Healey, K. R. et al. Prevalent mutator genotype identified in fungal pathogen *Candida glabrata* promotes multi-drug resistance. *Nat. Commun.* **7**, 11128 (2016).
159. Giraud, A. et al. Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science* **291**, 2606–2608 (2001).
160. McDonald, M. J., Rice, D. P. & Desai, M. M. Sex speeds adaptation by altering the dynamics of molecular evolution. *Nature* **531**, 233–236 (2016).
161. Rego, E. H., Audette, R. E. & Rubin, E. J. Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* **546**, 153–157 (2017).
This study shows that the heterogeneity of cell growth in mycobacteria is partially controlled by LamA, a divisome factor responsible for the asymmetric growth of daughter cells after cell division.
162. Jones, D. L., Brewster, R. C. & Phillips, R. Promoter architecture dictates cell-to-cell variability in gene expression. *Science* **346**, 1533–1536 (2014).
163. Dabrowski, M., Bukowy-Bieryllo, Z. & Zietkiewicz, E. Translational readthrough potential of natural termination codons in eucaryotes — The impact of RNA sequence. *RNA Biol.* **12**, 950–958 (2015).
164. Paul, K. R., Hendrich, C. G., Waechter, A., Harman, M. R. & Ross, E. D. Generating new prions by targeted mutation or segment duplication. *Proc. Natl Acad. Sci. USA* **112**, 8584–8589 (2015).
165. Zhang, Z., Qian, W. & Zhang, J. Positive selection for elevated gene expression noise in yeast. *Mol. Syst. Biol.* **5**, 299 (2009).
166. Wolf, L., Silander, O. K. & van Nimwegen, E. Expression noise facilitates the evolution of gene regulation. *eLife* **4**, e05856 (2015).
167. Osterwalder, M. et al. Enhancer redundancy provides phenotypic robustness in mammalian development. *Nature* **554**, 239–243 (2018).
168. Borenstein, E. & Ruppin, E. Direct evolution of genetic robustness in microRNA. *Proc. Natl Acad. Sci. USA* **103**, 6593–6598 (2006).
169. Toll-Riera, M., Bostick, D., Alba, M. M. & Plotkin, J. B. Structure and age jointly influence rates of protein evolution. *PLOS Comput. Biol.* **8**, e1002542 (2012).
170. Bloom, J. D. et al. Evolution favors protein mutational robustness in sufficiently large populations. *BMC Biol.* **5**, 29 (2007).
This article experimentally demonstrates that mutational robustness increases in the evolution of large populations.

171. Montville, R., Froissart, R., Remold, S. K., Tenaillon, O. & Turner, P. E. Evolution of mutational robustness in an RNA virus. *PLOS Biol.* **3**, e381 (2005).
172. Sanjuan, R., Cuevas, J. M., Furio, V., Holmes, E. C. & Moya, A. Selection for robustness in mutagenized RNA viruses. *PLOS Genet.* **3**, e93 (2007).
173. Szollosi, G. J. & Derenyi, I. Congruent evolution of genetic and environmental robustness in micro-RNA. *Mol. Biol. Evol.* **26**, 867–874 (2009).
174. Salverda, M. L. et al. Initial mutations direct alternative pathways of protein evolution. *PLOS Genet.* **7**, e1001321 (2011).
175. Chan, Y. H., Venev, S. V., Zeldovich, K. B. & Matthews, C. R. Correlation of fitness landscapes from three orthologous TIM barrels originates from sequence and structure constraints. *Nat. Commun.* **8**, 14614 (2017).
176. Haddox, H. K., Dingens, A. S., Hilton, S. K., Overbaugh, J. & Bloom, J. D. Mapping mutational effects along the evolutionary landscape of HIV envelope. *eLife* **7**, e34420 (2018).
177. Woods, R. J. et al. Second-order selection for evolvability in a large *Escherichia coli* population. *Science* **331**, 1433–1436 (2011).
178. Leon, D., D'Alton, S., Quandt, E. M. & Barrick, J. E. Innovation in an *E. coli* evolution experiment is contingent on maintaining adaptive potential until competition subsides. *PLOS Genet.* **14**, e1007348 (2018).
179. Gommans, W. M., Mullen, S. P. & Maas, S. RNA editing: a driving force for adaptive evolution? *Bioessays* **31**, 1137–1145 (2009).
180. Raman, A. S., White, K. I. & Ranganathan, R. Origins of allostery and evolvability in proteins: a case study. *Cell* **166**, 468–480 (2016).
181. Domingo, J., Diss, G. & Lehner, B. Pairwise and higher-order genetic interactions during the evolution of a tRNA. *Nature* **558**, 117–121 (2018).
182. Sailer, Z. R. & Harms, M. J. High-order epistasis shapes evolutionary trajectories. *PLOS Comput. Biol.* **13**, e1005541 (2017).
183. de Vos, M. G., Dawid, A., Sunderlikova, V. & Tans, S. J. Breaking evolutionary constraint with a tradeoff ratchet. *Proc. Natl Acad. Sci. USA* **112**, 14906–14911 (2015).
References 144 and 183 demonstrate that environmental change can facilitate adaptation by helping evolving populations escape the local optima of an adaptive landscape.
184. Ogbunugafor, C. B. & Eppstein, M. J. Competition along trajectories governs adaptation rates towards antimicrobial resistance. *Nat. Ecol. Evol.* **1**, 7 (2016).
185. Moratorio, G. et al. Attenuation of RNA viruses by redirecting their evolution in sequence space. *Nat. Microbiol.* **2**, 17088 (2017).
186. Prakadan, S. M., Shalek, A. K. & Weitz, D. A. Scaling by shrinking: empowering single-cell 'omics' with microfluidic devices. *Nat. Rev. Genet.* **18**, 345–361 (2017).
187. Crosetto, N., Bienko, M. & van Oudenaarden, A. Spatially resolved transcriptomics and beyond. *Nat. Rev. Genet.* **16**, 57–66 (2015).
188. Liu, S. & Trapnell, C. Single-cell transcriptome sequencing: recent advances and remaining challenges. *F1000Res* **5**, 182 (2016).
189. Ingolia, N. T. Ribosome footprint profiling of translation throughout the genome. *Cell* **165**, 22–33 (2016).
190. Hietpas, R. T., Jensen, J. D. & Bolon, D. N. Experimental illumination of a fitness landscape. *Proc. Natl Acad. Sci. USA* **108**, 7896–7901 (2011).
191. Thornton, J. W. Resurrecting ancient genes: experimental analysis of extinct molecules. *Nat. Rev. Genet.* **5**, 366–375 (2004).
192. Eyre-Walker, A. & Keightley, P. D. The distribution of fitness effects of new mutations. *Nat. Rev. Genet.* **8**, 610–618 (2007).
193. Eshel, I. Clone-selection and optimal rates of mutation. *J. Appl. Probabil.* **10**, 728–738 (1973).
194. Starrfelt, J. & Kokko, H. Bet-hedging — a triple trade-off between means, variances and correlations. *Biol. Rev.* **87**, 742–755 (2012).
195. Frank, S. A. *Foundations of social evolution* (Princeton Univ. Press, 1998).
196. Gardner, A., West, S. A. & Wild, G. The genetical theory of kin selection. *J. Evol. Biol.* **24**, 1020–1043 (2011).
197. Wilson, D. S. & Wilson, E. O. Rethinking the theoretical foundation of sociobiology. *Quarterly Rev. Biol.* **82**, 327–348 (2007).
198. Graves, C. J. & Weinreich, D. M. Variability in fitness effects can preclude selection of the fittest. *Annu. Rev. Ecol. Evol. S.* **48**, 399–417 (2017).
199. Griswold, C. K. & Masel, J. Complex adaptations can drive the evolution of the capacitor [*PSI*$^{+}$], even with realistic rates of yeast sex. *PLOS Genet.* **5**, e1000517 (2009).
200. Rutherford, S. L. Between genotype and phenotype: protein chaperones and evolvability. *Nat. Rev. Genet.* **4**, 263–274 (2003).

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