BETWEEN GENOTYPE AND PHENOTYPE: PROTEIN CHAPERONES AND EVOLVABILITY

Suzanne L. Rutherford

Protein chaperones direct the folding of polypeptides into functional proteins, facilitate developmental signalling and, as heat-shock proteins (HSPs), can be indispensable for survival in unpredictable environments. Recent work shows that the main HSP chaperone families also buffer phenotypic variation. Chaperones can do this either directly through masking the phenotypic effects of mutant polypeptides by allowing their correct folding, or indirectly through buffering the expression of morphogenic variation in threshold traits by regulating signal transduction. Environmentally sensitive chaperone functions in protein folding and signal transduction have different potential consequences for the evolution of populations and lineages under selection in changing environments.

CHAPERONES
A class of proteins that, by preventing improper associations, assist in the correct folding or assembly of other proteins *in vivo*, but that are not a part of the mature structure.

Division of Basic Sciences, Fred Hutchinson Cancer Research Centre, Mailstop A2–168, 1100 Fairview Avenue North, Seattle, Washington 98109–1024, USA.
e-mail: srutherf@fhcrc.org
doi:10.1038/nrg1041

The heat-shock proteins (HSPs) are highly conserved families of enzymes and CHAPERONES that are involved in the folding and degradation of damaged proteins. They are rapidly and concertedly mobilized in large numbers by cells that are under stress. The mobilization of HSPs is an important component of a universal and tightly orchestrated stress response that has probably allowed organisms to survive otherwise lethal temperatures throughout evolution[1,2]. Even at normal temperatures, several HSP chaperones are essential for viability, and promote the successful folding and activity of many cellular proteins[2–4]. Recent reports document further roles of some of the constitutively important chaperone families that are expressed at the population level[5–8]. Genetic or pharmacological manipulation of these chaperones alters the expression of genetic variation in several systems. Therefore, as well as having a vital role in stress physiology, chaperones also provide a plausible molecular mechanism for regulating the capacity of populations and lineages for evolutionary adaptation to changing environments — EVOLVABILITY.

It is thought that during periods of environmental stress, competition for chaperones by stress-damaged proteins compromises the ability of the chaperones to protect or fold their usual targets, thereby reducing the activities of most target proteins[9,10]. According to recent studies, the modulation of chaperone and target functions in response to stress would alternately mask and expose phenotypic variation, depending on the degree of stress and the availability of free chaperones[11–14]. This indicates that chaperones control a reserve of neutral genetic variation, which builds up in populations under normal conditions and could be expressed as heritable phenotypic variation during periods of environmental change. As the rate of evolution is limited by heritable variation in fitness, this chaperone-mediated mechanism might allow populations and lineages to better adapt to severe environmental change. The expression of random genetic variation is expected to be largely deleterious to individual fitness. However, both individual organisms and interbreeding groups of organisms produce the differential 'births' (new individuals or groups) and 'deaths' (loss of reproductive fitness or extinction) that are required for evolution. Under certain circumstances, population-level traits can increase group fitness more than they decrease individual fitness, even though the evolutionary forces that operate at each

Box 1 | **Regulated mutation**

Mutations provide the essential material for heritable change in biological evolution. Highly sophisticated DNA replication and repair machinery maintains genomic fidelity and, surprisingly, is also necessary for the generation of chemically or ultraviolet-light-induced mutations[83]. The elaborate molecular mechanisms that regulate mutation[84,85], and the constancy of per genome mutation rates across genomes of different sizes[86], indicate that mutation rates are optimized by natural selection. However, consideration of both the benefits of mutation in providing variation for adaptive evolution, and the deleterious fitness costs that most mutations incur, creates a tension between what is beneficial for the individual and what benefits the group. It is convincingly argued on population genetic principles that 'mutator alleles' of replication and repair genes cannot be maintained in most populations by the occasional selective advantage to the group of rare adaptive mutations[22]. The force of selection against mutator alleles is stronger at the level of the individual. Therefore, it is possible that mutation rates are kept as low as possible, given a trade-off between the overwhelmingly deleterious effects of mutations (MUTATION LOAD) and the 'energetic costs' of higher fidelity[22,87]. In this view, the benefits of mutator alleles in providing potentially fitter variants are transitory and depend on the population and the circumstances. For example, work by J. Arjan and G. M. de Visser in Richard Lenski's group delimits the utility of mutator genotypes in populations of asexual bacteria to small or initially well-adapted populations, in which the 'mutation supply rate' (the product of population size and mutation rate) is limiting[88].

Elegant biological solutions restrict elevated mutation rates to specific genetic loci or to temporary stress responses, and avoid many of the constraints that are associated with the evolution of unregulated mutator alleles[22]. So, the transient induction of error-prone DNA polymerases in response to the stress of an altered adaptive landscape is plausibly an evolved mechanism that increases the generation of new mutations at the time when they might be most useful, providing an enhanced opportunity for adaptive evolution and survival[89–91]. In some instances, error-prone polymerases have been co-opted to an evolutionary group advantage. For example, the targeted somatic hypermutation of immunoglobulin genes in B-cell lines is achieved in humans by a homologue of the yeast RAD30 highly-error-prone polymerase[92]. Gene-specific mutators, such as target genes that encode the coat proteins that allow some pathogenic bacteria to evade immune responses, and the enhanced mutation rates in immunoglobulin genes that allow them to target rapidly evolving pathogens, are clear examples of adaptively regulated evolvability that benefits populations of pathogenic bacteria or antibodies[93].

EVOLVABILITY
The ability of random genetic variation to produce phenotypic changes that can increase fitness (intrinsic evolvability) or the ability of a population to respond to selection (extrinsic evolvability). Extrinsic evolvability depends on intrinsic evolvability, as well as on external variables such as the history, size and structure of the population.

GROUP SELECTION
Selection on traits that increase the relative fitness of populations or lineages of organisms at some fitness cost to individuals. All of the feasible mechanisms require selection on lineages or small interbreeding groups of related individuals in subdivided populations.

MUTATION LOAD
The accumulated deleterious alleles that are carried by a population at any given time.

EXPRESSED MUTATION RATE
The rate of phenotypic change that results from the continuing accumulation of new mutations (expressed mutation rate = total mutation rate – neutral mutation rate).

THRESHOLD TRAITS
Quantitative traits that are discretely expressed in a limited number of phenotypes (usually two), but which are based on an assumed continuous distribution of factors that contribute to the trait (underlying liability).

level differ substantially in timescale and strength (for example, frequent and strong selection on individuals, compared with less frequent and weaker selection on large, randomly mating populations)[15]. Evolvability could have arisen either through selection for its benefits to small or highly structured populations or lineages — GROUP SELECTION — or as an unselected consequence of adaptive or neutral traits in individuals[16].

Historically, the discussion of regulated evolvability has centred on the regulation of mutation and recombination (BOX 1). The conditions under which the increased generation of variation by mutation is adaptive to populations and lineages under selection have been considered in depth with respect to the evolution of 'inducible mutators', which increase the mutation rate in response to stress. Many of the same issues apply to the evolution of chaperone-regulated evolvability, although — in contrast to the inducible mutators — chaperones do not alter genotype, but rather the expression of genetic variation as phenotypic variation. This review considers the possibility that the regulation of the EXPRESSED MUTATION RATE and THRESHOLD TRAITS by molecular chaperones is potentially adaptive, and that group and lineage level selection could, therefore, have shaped the properties of chaperone buffering systems.

In 1998, we reported that the signal-transduction chaperone Hsp90 regulates heritable morphological transitions in *Drosophila*, indicating a stress-sensitive biochemical mechanism that — because it produced conformational switches of signalling proteins in millisecond timescales — had the potential to influence the trajectories of developmental programmes over evolutionary time[6]. This work attracted the attention of biologists ranging from protein biochemists to ecologists and evolutionary biologists[17–20]. Recent experiments indicate that protein folding by chaperones in the HSP70 and HSP60 families also buffers phenotypic variation. Interest in evolvability, combined with a growing acceptance of group selection as a potentially powerful evolutionary mechanism[15,16,21–25], now encourages a re-examination of chaperone biology that considers its evolutionary consequences.

From folding to function

Chaperones act between the genotype and the phenotype to affect the expression of genetic variation. A growing number of studies have examined the role of chaperones in regulating the expression of mutations and genetic variation at different levels of phenotype. This review is organized around the successively higher levels of phenotype that have been examined (FIG. 1). As discussed below, protein-folding chaperones directly influence target-protein activity by allowing structurally unstable mutant protein sequences to fold into active configurations.

Protein folding in vivo. Protein folding, maintenance and repair are highly specialized cellular functions[2–4]. From nascent chains to progressively more mature folding intermediates, or from inherently unstable mutant proteins to those damaged by stress, abundant chaperones protect cellular proteins that otherwise would be prone to aggregate — allowing them to fold *de novo* or to refold after stress. Chaperones act by preventing the

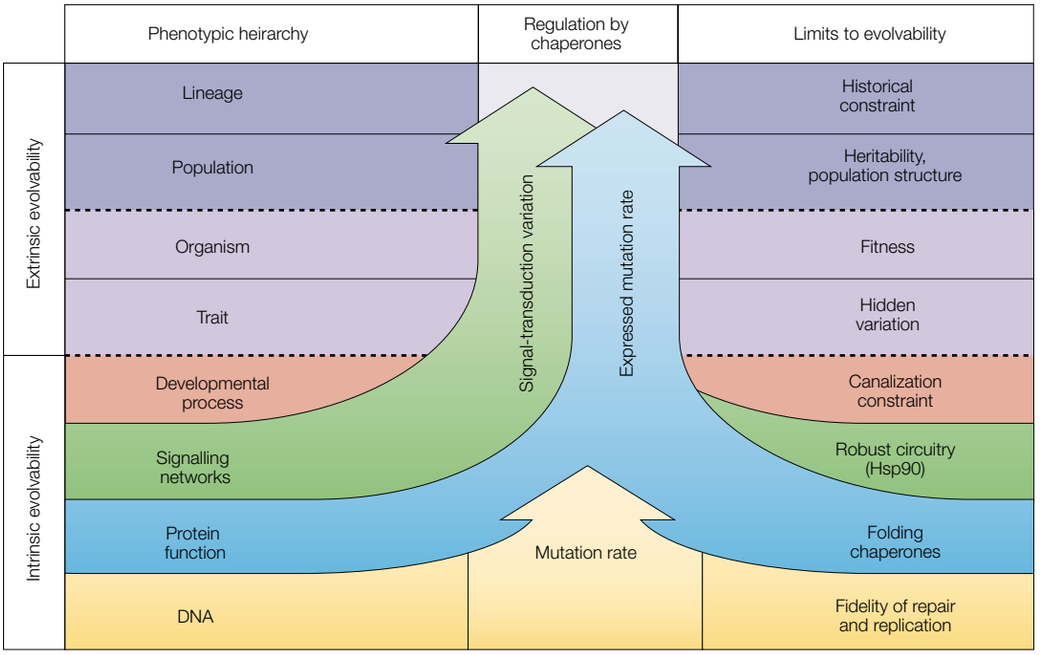


Figure 1 | **Hierarchical constraints on evolvability.** To influence evolution, the impact of mutations must be propagated through successively higher levels of phenotype (left column) to affect fitness. Evolvability is regulated at the levels of DNA mutation (yellow) and protein folding (blue), and by the strength and connectivity of Hsp90-dependent signalling pathways (green). Variation is detected at many levels of phenotype, from protein function to signalling processes, traits, populations and beyond. The factors that limit the expression of variation at each level are indicated on the right. Below the heavy dashed lines, developmental rules based on molecular mechanisms — such as chaperone-mediated protein folding — predominate (intrinsic evolvability). Above the heavy dashed lines, evolutionary mechanisms based on selection on populations predominate (extrinsic evolvability). In the transition zone between the dashed lines, organisms and traits have molecular, developmental and population-level properties. Events at successively higher levels can dampen the impact of mutations and variation in lower levels.

formation of promiscuous, but energetically stable, associations in, or between, non-native polypeptides; many use ATP-driven cycles of binding and release to destabilize non-native intermediates. This gives the polypeptides repeated opportunities to reach a stable mature fold.

The main HSP families recognize different structural features that are specific to immature, unstable and damaged proteins (TABLE 1). As discussed below, the HSPs that are constitutively involved in protein folding and maturation (HSP60, HSP70 and HSP90) also buffer phenotypic variation, whereas the small HSPs and HSP100 are primarily involved in recovery after stress, are not reported to buffer variation and, therefore, are not considered further here. The Hsp60s comprise small families of multimeric barrel-shaped proteins (chaperonins) with an interior folding cavity. The Hsp60/GroEL::Hsp10/GroES complex folds many bacterial proteins, whereas the eukaryotic tailless complex polypeptide-1 (TCP-1) ring complex (TriC) folds a more limited set of cellular targets[2,3]. In eukaryotes, HSP70 is relatively more important for general protein folding. The HSP70s are a large family of highly related proteins with chaperone activity that is used for diverse cellular functions in addition to *de novo* folding and recovery, including assisting with the post-translational unfolding and translocation of nuclear-encoded proteins through the lipid bilayers of organelles[4] and the assembly of HSP90 signalling complexes[26]. Unlike Hsp60 and Hsp70, Hsp90 is not involved in normal protein folding[27]. Hsp90 recognizes the hydrophobic surface features that are found uniquely on a limited set of nearly mature, but inherently unstable, signalling proteins in normal conditions[28,29], and that are found generally in the initial stages of unfolding on proteins that have been damaged by stress[30,31]. Although the HSP60/chaperonins and HSP70 fold many different general targets, the HSP90 targets are normally highly specific cell-cycle regulators and developmental regulators such as cyclin-dependent kinases, tyrosine kinases and certain classes of transcription factor[28,32]. These inherently unstable signalling targets are probably recognized by HSP90 on the basis of the non-native features that are common to proteins that function as molecular switches[29]. The distinct role of HSP90 in signal transduction is highlighted by its specific genetic interactions. Hsp90 has been found in several signal-transduction pathways in many large genetic-interaction screens[11–14]. The well-studied synthetic interactions between HSP90 and signalling mutants are a model for the HSP90 buffering of the natural polygenic variation that affects the signalling pathways that underlie threshold traits[14] (BOX 2).

Table 1 | **The main HSP chaperone families**

Chaperone	Binding topology	Action	References
HSP100		Disaggregates	79,80
HSP90		Supports nearly mature conformations; signal transducers	11,12
HSP70		Binds linear polypeptide for folding; translocation assembly of multiprotein complexes	3,4
HSP60 (GroEL, TriC)		Folds 'molten globule' proteins or domains	2,4
Small HSPs		Prevents aggregation, particularly during heat shock	1,3

Different chaperone families recognize structural features that are specific to immature and unstable proteins at different stages of folding and unfolding. The HSP70 chaperones, ring-shaped chaperonins and HSP90 are involved in successively more advanced stages of protein folding and function, whereas the small HSPs and HSP100 act predominantly during stress[112,113]. Adapted with permission from Table 1 in REF. 4 © (1998) Cell Press. HSP, heat-shock protein.

Masking mutations. Protein targets differ in their dependence on chaperones — a dependence that is increased by destabilizing mutations in the targets. For example, although HSP90 targets *in vivo* are highly specific, if HSP90 is added to many different denatured proteins *in vitro* it acts as a general chaperone[33,34]. Genetic studies of protein folding in yeast also indicate that unstable mutant proteins probably require HSP90 for proper folding and subsequent activity *in vivo*, whereas their more stable counterparts are independent of the chaperone[27,35]. Similarly, the overexpression of GroEL::GroES suppresses destabilizing mutations in diverse genes in *Escherichia coli*, restoring their activity[36]. So, chaperones seem to interact directly with particular mutant alleles, masking their effects by helping proteins to fold despite their mutations. If protein folding by chaperones becomes limiting — as might occur under severe protein stress — the expressed mutation rate of nonfunctional and partly functional proteins is increased, as illustrated by the hierarchical model in FIG. 1.

From sequence to structure

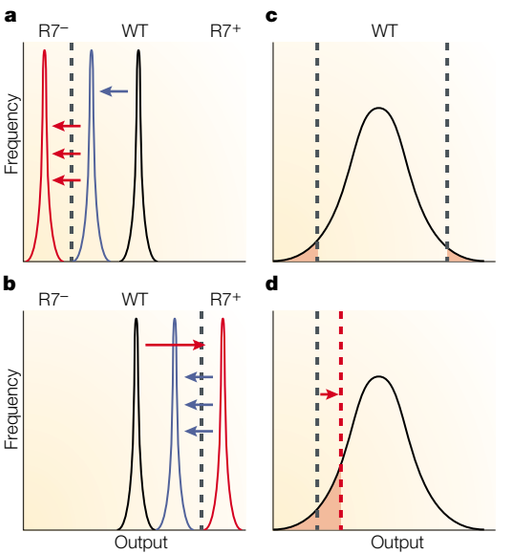
Protein-folding chaperones facilitate the transition from primary protein sequence to folded structure, and help alternative sequences — which would otherwise fold improperly — to reach a final stable conformation. The ability of chaperones to expand the number of viable sequences that can map to a single functional structure is theoretically important for the evolution of protein structural complexity.

The topology of sequence space. The mutational neighbourhood of a protein or RNA sequence is the family of sequences that are one or a few mutational steps away from the primary sequence. As proteins evolve through the sequential accumulation of amino-acid changes, each successive amino-acid sequence and structure determines the neighbourhood of viable mutational options available to them. The conserved structure and function of many protein families with evolutionarily diverged primary sequences[37], and the common ability of many proteins to tolerate amino-acid sequence changes without disturbing function, show that protein sequences can form extensive mutational neighbourhoods that map to identical structures. Extended mutational neighbourhoods of alternative primary sequences that map to the same structures are called 'neutral networks'[38].

The evolutionary consequences of extended multidimensional neutral networks have been studied in simulations of RNA secondary-structure 'phenotypes' under selection[39–41]. For example, the ability of RNA primary sequences to adopt secondary structures is based on biophysical principles, according to which each primary sequence can be represented by a repertoire of energetically stable shapes. Populations of RNA molecules 'explore' sequence space by random diffusion on the neutral network of their presently most viable phenotype, which enables well-defined and localized subpopulations to disperse without changing structure[40]. Meyers and Fontana discovered that populations of RNA molecules that evolve towards a target shape become trapped in regions of their neutral network where sequences encode the most thermodynamically stable, and 'fittest' structures[41]. These stable sequences are less likely to encode altered structures when mutated. Consequently, in this system, evolved structural robustness decreased the genetic variability (the capacity to express genetic variation), and, therefore, the evolvability of the system. Interestingly, the principles determined *in silico* for RNA secondary structure also apply to the fitness landscapes of RNA viruses undergoing laboratory evolution[42].

Evolvability of structure. Diffusion in sequence space allows populations of primary sequences to gain access to alternative neutral networks of specific new structures within a few mutations of the dominant phenotype. In the absence of extended neutral networks, a population

Box 2 | **Hsp90 maintains trait thresholds**



Molecular genetic screens show the existence of thresholds that determine phenotypic transitions in development, and show that HSP90 protects trait thresholds against the expression of previously silent genetic variants that affect signal-transduction pathways. For example, a well-characterized mitogen-activated protein kinase (MAPK) signalling pathway initiated at the *Drosophila sevenless* (*sev*) receptor determines photoreceptor R7 fate and is a model for Hsp90 interactions with threshold traits. High-level activation of the *sev* MAPK pathway ('output' in figure) promotes the differentiation of the R7 photoreceptor (WT, wild-type; $R7^+$, extra R7), whereas lower-level activity of the cascade results in a default non-neuronal cell fate among the equipotent cells of the R7 equivalence group ($R7^-$). In a series of genetic screens that have been instrumental in identifying the downstream components of receptor tyrosine kinase signalling, endogenous components of the *sev* cascade have been replaced with marginally underactivated or overactivated analogues[94–96], shown with the transition from the normal (black) to the sensitized (blue) distributions in panel **a**, and the sensitized and mutant (red) distribution in panel **b**. Heterozygous recessive mutations in Hsp90 or other pathway components compensate[11–13,97], bringing flies below the lower threshold to express the $R7^-$ phenotype (for example, the red arrows and distribution in panel **a**) or below the upper threshold from a mutant ($R7^+$) to a normal phenotype (for example, the blue arrows and distribution in panel **b**). The heterozygous enhancer and suppressor mutations would normally be cryptic and not phenotypically expressed, but if they were combined with the previously cryptic sensitized genetic backgrounds, they would alter the phenotype. Hsp90 is identified in these pathways, along with specific downstream effectors such as RAS and the RAF MAPK (REFS. 13,97), probably because it supports RAF activity[11,98,99]. The role of Hsp90 in acting through RAF to maintain thresholds for R7 determination is a model for its role in the signalling pathways that are governed by its targets.

The success of sensitized genetic screens depends on the uniformity of strain backgrounds and their signalling 'outputs' (narrow distributions in panels **a** and **b**). Experiments in which the *sev* pathway was sensitized in a set of wild-type genetic backgrounds show that, in outbred flies, there is a surprisingly wide range of naturally occurring variation with both positive and negative effects on the output of *sev* signalling, which exceed the effects seen by the main loss-of-function mutations in the laboratory strains[59]. In outbred wild populations, the distribution of pathway outputs is expected to be wider and maintained by stabilizing selection for an intermediate output that avoids the aberrant phenotypes beyond the trait thresholds at either extreme (panel **c**). If Hsp90 is impaired in wild backgrounds (panel **d**), several signalling pathways are 'sensitized' through the decreased activity of its targets, effectively shifting the trait thresholds to the right (red arrows). More individuals with low signalling output now express abnormal traits (red fill). Under low Hsp90, marked morphological variation that affects threshold traits is expressed[6]. Depending on the specific genetic background, this affects many different adult structures. Rather than resulting from heterozygous single-gene mutations, this naturally occurring background-specific variation is polygenic, and depends on the interactions of several loci throughout the genome and their response to the environment. In the molecular genetic framework exemplified by the *sev* pathway, Hsp90 maintains thresholds above or below which Hsp90-dependent pathways fail.

Once traits are expressed, selection enriches genetic determinants for the trait in the selected populations until their frequency is so high that the threshold is surpassed in most individuals and the trait is expressed independently of the Hsp90 mutant in which it was initially discovered[6]. This models the possible evolutionary transition to adapted phenotypes under sudden environmental change. Initially, the stress of environmental challenge causes protein damage and a stress response. If the stress is sufficiently severe, chaperones are overwhelmed and previously silent variations become available for selection. As protein folding and metabolism becomes adapted to the new environment, or if the environment returns to its previous norms, damage is curtailed and the stress response subsides; at the same time, potentially adaptive new morphological phenotypes become fixed by selection. This process would allow new adaptive phenotypes to become constantly expressed, even in the absence of stress. Figure reproduced with permission from REF. 14 © (2002) Wiley-Liss, Inc.

would be unable to explore a large number of new and potentially adaptive structures. As discussed above, in stabilizing otherwise unstable proteins, chaperones increase the number of polypeptide sequences that can attain the same structure — by definition, expanding mutational neighbourhoods and neutral networks in protein sequence space. If the general principles that have been developed for the evolution of RNA secondary structure also apply to the more complex evolution of protein structures, then chaperone action would influence the evolvability of protein structures. Based on the RNA studies, it might be expected that chaperones

would either increase evolvability by preventing populations of sequences from becoming entrenched in regions of sequence space where their capacity to evolve is curtailed, or that they might decrease evolvability by promoting evolved structural robustness. As evolvability results from a balance between robustness and variability (BOX 3), the consequences of chaperones for protein structure evolution will be complex. Our limited ability to predict protein structure does not yet allow the detailed modelling that has been carried out with RNA secondary-structure neighbourhoods. However, the RNA studies highlight the importance of understanding the co-evolution of complex proteins with their chaperone counterparts.

From function to fitness

The role of chaperones in buffering conformation and function is translated from protein activity to higher-level phenotypes — for example, the competitive fitness of bacteria with accumulated deleterious mutations and morphological phenotypes that result from natural stresses in *Drosophila* in the wild.

Compensatory buffering. Even if mutations cause a catastrophic failure in protein folding, they do not necessarily affect fitness, as evidenced by the large number of genes, in many organisms, that can be deleted without apparent consequences for viability[43]. Nevertheless, in the absence of mating and recombination, GENETIC DRIFT in small populations results in the accumulation of deleterious mutations and the random, but inescapable, loss of the fittest genotypes[44]. The lifecycle of ENDOSYMBIOTIC BACTERIA in separate hosts results in small clonal populations that are expected to evolve progressively deteriorated fitness[45]. However, this seems not to occur. The GroEL::GroES folding machine is constitutively upregulated in most lineages of the aphid endosymbiont *Buchnera*, accounting for as much as 10% of total protein in some cases[46]. In 1996, Moran suggested that the massive constitutive overexpression of GroEL found in some endosymbionts is an evolutionarily compensatory mechanism that allows proteins to retain function despite the accumulation of amino-acid changes[46]. In contrast to the accumulation of non-synonymous genetic changes in *Buchnera* lineages of different hosts, the GroEL gene accumulated relatively few non-synonymous substitutions[47,48]. This implies that functional changes in GroEL are not tolerated, and are removed from the population by PURIFYING SELECTION. The most direct evidence for the role of the GroE operon in buffering deleterious variation comes from recent mutation accumulation experiments in *E. coli*, a close relative of *Buchnera*. Overexpression of GroE significantly restored fitness to mutated strains after more than 3,000 generations of random mutation accumulation[5]. Taken together, the evidence from *E. coli* and related pathogenic isolates indicates that the GroE operon restores fitness by directly buffering the effects of the accumulated deleterious alleles. The capacity of overexpressed GroE products to buffer detrimental mutations is a reflection of the expanded 'folding landscape' — as produced by chaperones — and the importance of genetic buffering to individual fitness, particularly for small clonal populations.

Heat shock in nature. The heat-shock response is remarkably adapted to the specific ecologies of different organisms, ranging from subtidal algae (which induce a heat-shock response at 5°C) to the extreme hyperthermophylic Archaea (which induce a heat-shock response at temperatures >100°C)[1]. Data indicate that the heat-shock response is highly plastic in its evolutionary adaptation to different environments — even in the same species. For example, the threshold induction temperature of HSPs in two species of goby fish showed a seasonal acclimatization, increasing in temperature by several degrees centigrade in the summer compared with winter HSP induction temperatures[49]. Laboratory experiments also indicate that abundant chaperones, which are either constitutively present or induced by stress, recognize features that are characteristic of stress-damaged and unfolded proteins and facilitate their refolding or degradation, to enable survival. However, a detailed knowledge of the frequency and importance of the heat-shock response to most organisms in nature is lacking. An elegant body of work from the laboratory of Martin Feder on the role of *Drosophila* Hsp70 in the ecology and evolutionary physiology of stress tolerance provides important natural context to our predominantly laboratory-based understanding of the heat-shock response.

Hsp70 ecology and physiology. *Drosophila melanogaster* in the wild typically feed on damaged and fermenting fruit. In direct sunlight, fruit-containing *Drosophila* larvae can reach ambient temperatures as high as 44°C — although 30°C is the highest sustained temperature that is compatible with the growth and reproduction of flies in the wild[50]. If the temperature reaches ~36°C, the rate at which Hsp70 is transcribed increases greatly, increasing the previously negligible levels of the product to 1–2% of the cellular protein in a matter of minutes[1]. Field studies have indicated that there is a direct relationship between Hsp70 activation, survival and fitness in flies in the wild. To investigate the functional correlation between Hsp70 induction and stress tolerance in *Drosophila*, experimental lines of flies were engineered to contain 12 extra transgenic copies of the Hsp70 gene[51] (extra-copy lines). When placed under natural thermal stress, comparison with perfectly matched excision controls (excision lines) — containing only the disrupted transgene insertion site — showed markedly increased stress tolerance in the extra-copy lines[52]. Importantly, both extra-copy lines and excision lines had the same uniform genetic background, so any differences in the physiology of heat tolerance could be attributed solely to the Hsp70 gene, and not to variation in the myriad other HSPs or physiological factors, or to mutational effects of the transgene insertion site. Furthermore, protection against heat stress in this system was paralleled with the ability to reactivate a native protein template,

GENETIC DRIFT
The stochastic change in gene frequency that results from binomial sampling of alleles segregating in finite populations.

ENDOSYMBIOTIC BACTERIA
Non-pathogenic bacteria that live inside host cells.

PURIFYING SELECTION
Selection against deleterious alleles that arise in a population, preventing their increase in frequency and assuring their eventual disappearance from the gene pool.

alcohol dehydrogenase (Adh)[53]. After a 40°C heat pulse, Adh activity in fly extracts declined to <50% of its pre-pulse levels; this activity was restored in 2 hours in the extra-copy lines, but remained at the same low level in the matched excision-line controls. Therefore, thermotolerance in *Drosophila* is primarily attributed to Hsp70, and is correlated with its ability to restore the function of heat-damaged proteins.

Stress and variation in the wild. In a significant extension of this work, Roberts and Feder reported that natural heat-stress encountered by wild *Drosophila* larvae leads to a PHENOCOPY of developmental defects in >10% of surviving adults[8]. Remarkably, surviving adults derived from transgenic extra-copy Hsp70 larvae, which had been similarly exposed to heat, had fewer morphological abnormalities. Phenocopies that affect specific developmental features have long been studied in the laboratory by applying particular stresses during precise windows of development[54]. The laboratory expression of phenocopy is influenced by genetic variation[55]. However, the relative extent to which natural genetic and environmental factors contribute to the morphological variants that are masked by Hsp70 is not yet known, neither is it known whether Hsp70 buffering results from its role in protein folding, environmental stress protection or as a co-chaperone for Hsp90-dependent signal transduction (see below). On the basis of the importance of Hsp70 in the folding and reactivation of damaged targets, it is likely that Hsp70 is limiting

Box 3 | **Measuring evolvability**

Evolvability — the ability to produce evolutionary 'improvement' or adaptation through the process of random mutation, recombination and selection[100] — is most easily observed at the level of species, as seen, for example, in the extensive ADAPTIVE RADIATIONS of certain CLADES[15]. Although comparing the relative diversification of different lineages is conceptually simple, to be reliable, these comparisons require a substantial fossil record. A more problematic issue is that evolvability itself, as the capacity for evolutionary adaptation, is not predictive of diversification. So, meaningful comparisons between taxa must control for the external factors, such as selection pressures, life history and ecology, that influence the rate and extent of species diversification[101]. The expression of variation in populations begins with individual development, which roots evolvability in molecular mechanisms. It is useful, therefore, to consider evolvability seperately at the level of populations ('extrinsic') and at the level of individual development ('intrinsic').

Extrinsic evolvability

Extrinsic evolvability is defined as the ability of populations to respond to selection[102]. However, as with all evolutionary mechanisms that act on populations, extrinsic evolvability is influenced by external variables that determine the rate of genetic change. For example, both population structure and EFFECTIVE POPULATION SIZE have a crucial impact on the rate of evolution, which is defined by changing gene frequencies[23,25,103]. In turn, gene frequencies determine evolutionary rates by affecting both the expression of genetic variation by a population and the HERITABILITY of selected traits[104]. So, although extrinsic evolvability can be readily measured in the response of populations to selection, the important parameters are not innate, and change as selection progresses.

Intrinsic evolvability

Intrinsic evolvability directly affects the expression of heritable variation. It is innate, and has two distinct and important components[100]: variability (the ability to produce heritable phenotypic variation in response to genetic changes) and selectability (the probability that random phenotypic variants will be functionally adaptive and without correlated negative fitness costs[16,20,105]). Both protein-folding chaperones and signal-transduction chaperones regulate variability; however, their influence on selectability is more controversial, as it has been argued that the phenotypic variation buffered by chaperone function is unconditionally deleterious[18,20,106]. Computer modelling and design principles show that random structural alterations to a program or structure can be unconditionally deleterious without the possibility for improvement, and that they depend on properties of the system architecture that bias random changes towards useful variation (selectability). These properties are, therefore, a crucial component of evolvability[100].

Selectability

The best understood, and perhaps the most important, feature that facilitates selectability is modular design, which restricts the effect of mutational changes and allows isolated traits or groups of related traits to be modified autonomously. Some types of modularity are innate to developmental processes in individuals[16]. However, modularity can also be defined by extrinsic population-level parameters such as trait covariances, which depend on allele frequencies in populations. The covariance of traits with one another and with fitness is one measure of their modularity — traits with low covariance might be more easily modified by selection. Ironically, all else being equal, both robustness (decreased variability) in modules and variability between modules can be crucial for modularity[16]. Population-genetic models also indicate that evolvability stems from the tension between variability and modularity[107]. Implicit in these arguments is the fact that the particular arrangement of traits into modules is determined by selection, either for group evolvability or for the physiological flexibility it confers on individuals[16]. It is therefore possible that many organizational and design principles that allow genotypes to produce potentially adaptive variation are universal features of life and, therefore, would be difficult to observe.

PHENOCOPY
The production of a phenotype as a result of environmental factors, such as stress, which closely resembles a phenotype that normally results from specific gene expression or from gene mutation.

ADAPTIVE RADIATION
The expansion of a lineage into a group of new lineages by speciation. It is often associated with the exploitation of new ecological niches.

CLADE
A group of species that has diversified from a common ancestor.

EFFECTIVE POPULATION SIZE
(N_e). The theoretical size of an idealized population for which the genetic variation in a sample is explained solely by mutation and genetic drift.

HERITABILITY
The proportion of total phenotypic variation that can be attributed to genetic effects (broad sense) or purely additive genetic effects (narrow sense). Narrow-sense heritability predicts the initial response of a population to selection and decreases over the course of selection.

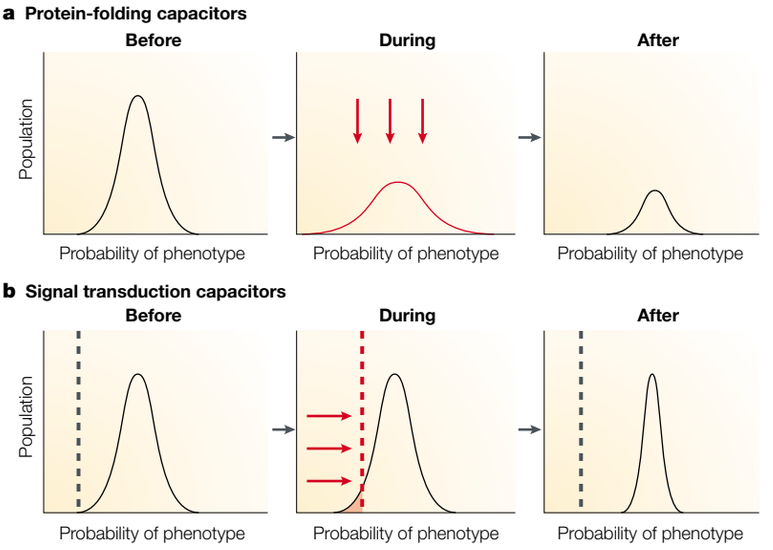


Figure 2 | **Group fitness and evolutionary capacitors.** Theoretical population-level fitness effects of protein-folding capacitors compared with signal-transduction capacitors in response to an episode of stress and selection on a threshold trait. Populations are assumed to have been previously optimized at the population mean (underlying liability) by stabilizing selection. Distributions before, during and immediately after previously hidden variants that are buffered by protein-folding chaperones (panel **a**) or signal-transduction chaperones (panel **b**) are exposed by stress (red arrows and red fill). After the episode of stress and selection, the fitness of the upper right population is degraded, whereas the fitness of the lower right population is intact.

in the reactivation or protection of developmentally important proteins that have been damaged by heat, and that the decreased function of these developmental proteins is responsible for the phenocopies. Regardless of the precise mechanism, the *Drosophila* Hsp70 story shows the importance of chaperones for survival in the wild, and provides an exciting link between molecular chaperones, buffered morphological variation in wild populations and natural environmental stress.

From signal transduction to threshold traits

If HSP90 is impaired in cells, signal-transduction targets begin to lose function[11,13,35,56,57], and complex morphogenic variation for threshold traits is exposed[6,14] (BOX 2). In contrast to buffering by protein-folding chaperones — which directly buffer mutations in target proteins, as described above — much HSP90 buffered variation is probably a secondary consequence of the weakening of signal-transduction targets. For example, when genetically weakened ('sensitized') targets in Hsp90-dependent eye-development pathways were placed in different natural genetic backgrounds, potent variation was uncovered in the wild-type flies[58,59]. So, although amino-acid replacements and coding mutations would be buffered by protein-folding chaperones, any type of genetic mutation that affects the strength of signal transduction through Hsp90-dependent pathways ('output') (BOX 2) could be buffered by the Hsp90 system (coding, regulatory, point mutation, chromosomal rearrangement and transposition).

STABILIZING SELECTION
Selection for an intermediate phenotypic optimum that is fitter than either higher or lower phenotypes.

Surprising morphological novelty. It is estimated that <12% of *Drosophila* genes are singly mutable to altered morphology in viable adults[60]. Furthermore, only a small fraction of these 'major effect' mutations yield competitively fit animals, owing to the crucial role of these genes in development. In view of the apparently limited capacity of mutations to alter morphology, it is remarkable that Hsp90 buffers the expression of morphological transitions in a variety of organisms. These include morphological novelties in laboratory and wild populations of *Drosophila*[6] and *Arabidopsis*[7], cortical patterning in *Tetrahymena*[61,62], developmental defects in fungi[63–66] and metamorphosis in Ascidian embryos[67,68]. In contrast to the 'major effect' mutations in single genes, the large morphological changes associated with new *Drosophila* phenotypes are polygenic and can be selected under low Hsp90 function without apparent correlated fitness effects (S.L.R., unpublished observations). Through laboratory selection, lines of flies can be generated in which most individuals express the abnormal trait. Selection enriches lines for trait polymorphisms to the point where the threshold is surpassed and the trait is expressed independently of the Hsp90 mutant in which they were first found [6,14] (BOX 2). In theory, if several polygenic alleles interchangeably produce the trait under selection, the most deleterious polymorphisms can be lost during selection, whereas the phenotype is maintained by alternative — and less deleterious — trait polymorphisms.

Evolutionary capacitors. Positioned between the source of heritable variation (genotype) and its expression (phenotype), chaperones allow cryptic variation to accumulate in phenotypically normal populations. If chaperone buffering is overwhelmed, the stored genetic variation is expressed as phenotypic variation, inspiring the capacitor metaphor[6]. However, determining if, and how, the concerted release of phenotypic variation under environmental stress would influence the course of evolution, requires consideration of the population-level consequences of stress-sensitive genetic buffering.

Consider hypothetical trait distributions in a previously well-adapted population before, during and after an episode of stress that exposes chaperone-buffered variation. Importantly, the theoretical population-level consequences of regulated genetic buffering by protein-folding chaperones compared with signal-transduction chaperones (such as Hsp90) are distinct (FIG. 2). Chaperones that are limiting for protein folding — such as GroEL::GroES — modulate the activities of variant proteins directly, and, therefore, regulate the expressed mutation rate (upper panel in FIG. 2). For a population that has been previously optimized by STABILIZING SELECTION to have an intermediate and optimal phenotype in the environment before selection, all genotypes — including the previously most well-adapted — are degraded during stress by the increased expression of pre-existing mutations, most of which will be deleterious. In this case, the population consequence of limiting chaperone function is the same as the consequence of increasing the mutation rate: to

widen the distribution of phenotypes providing the opportunity for potentially beneficial mutations in rare individuals, but generally degrading the average fitness of the population.

In a crucial distinction from the 'protein-folding capacitors', 'signal-transduction capacitors' (such as HSP90) buffer variation by maintaining trait thresholds. If HSP90 is impaired during stress, thresholds are shifted, affecting only the individuals that are already at the most extreme tails of the distribution (lower panel in FIG. 2). Because only individuals that carry the most extreme genotypes are at risk for expressing abnormal phenotypes, the consequences for the population are similar to the effects of stabilizing selection (lower right panel in FIG. 2). During, and after, the episode of stress, cryptic variation remains concealed in the most adapted genotypes (centre of the distribution in FIG. 2). Because they are not subject to purifying selection, the fittest genotypes in the population are conserved. After the episode of stress, the previously optimized phenotype — and the overall fitness of the population — are maintained (FIG. 2). So, relief from Hsp90 buffering under stress would allow populations to test morphological novelties in extreme individuals, without the entire population experiencing the generally detrimental effects of increased mutations.

Evolved evolvability? Development is highly buffered in many ways against small genetic and environmental perturbations, as a result of several molecular mechanisms and evolutionary pressures[14,16,69,70] (FIG. 1). For example, the connectivity, complexity and strength of signal-transduction pathways determines their capacity to vary in response to mutation[71]. Through its stress-regulated role in supporting several specific signalling targets, HSP90 might be uniquely positioned to destabilize the otherwise entrenched outcomes of complex networks of developmental pathways (FIG. 3). By simultaneously modulating the activities of several developmental pathways, HSP90 could synchronously expose polygenic

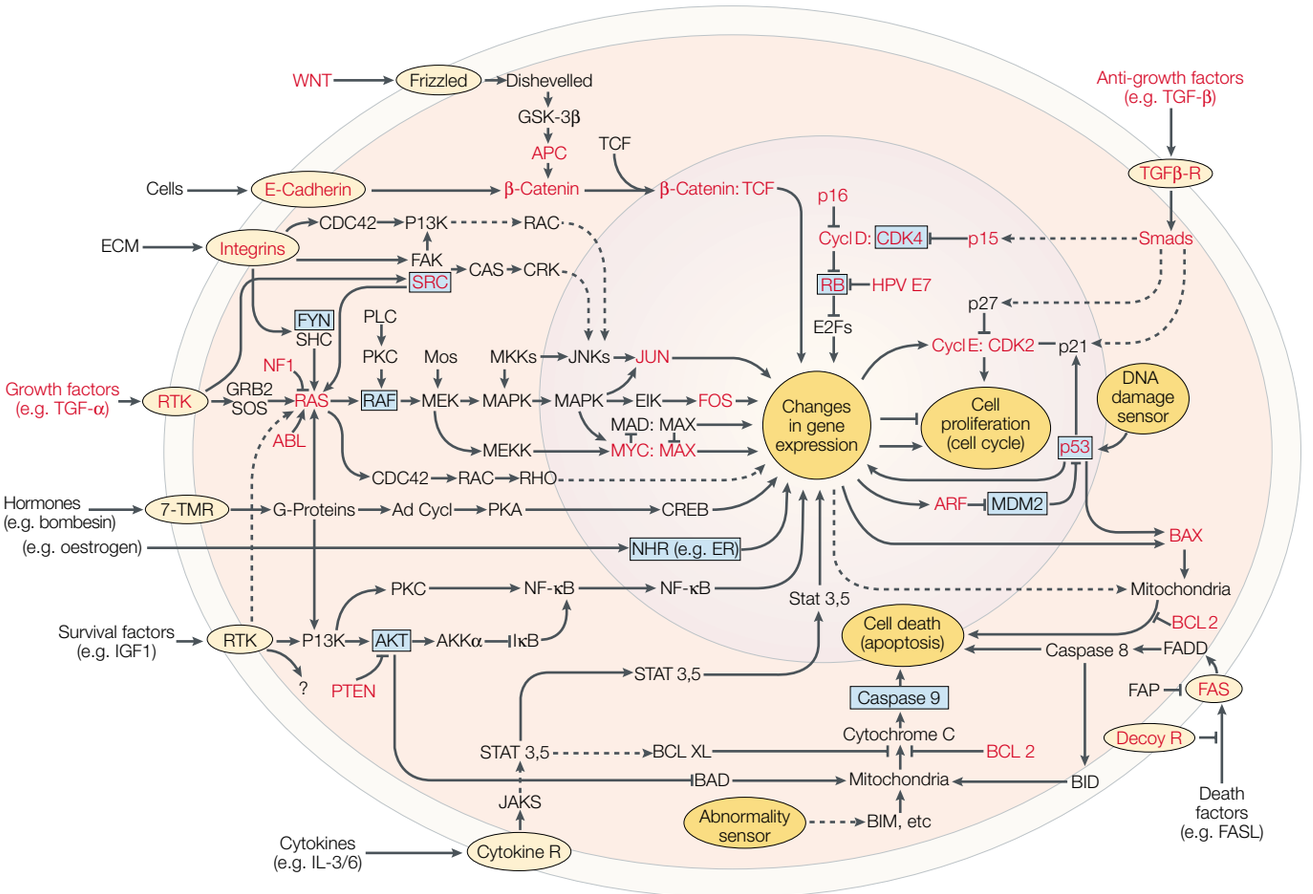


Figure 3 | **HSP90 targets in a signalling network.** Complex and interconnected signalling networks allow cells to integrate information that regulates the decision to grow, differentiate, divide or undergo programmed cell death. Stress could simultaneously destabilize HSP90 targets (blue boxes), disrupting the rate-limiting steps, parallel pathways and feedback loops that buffer complex networks against change[71]. HSP90 targets are as in REFS 28,29,32. Other targets (not included in these references) are AKT (REF.108), procaspase-9 (REF. 109), MDM2 (REF. 110) and PI3K, which is indirectly affected through the HSP90 target PDK1 (REF. 108). Tumour suppressors and oncogenes are shown in red. Broken lines denote mulltiple steps. ECR, extracellular matrix; ER, oestrogen receptor; R, receptor. Other abbreviations as in REF. 111. Modified with permission from REF. 111 © (2002) Cell Press.

SPANDRELS
Features that arise as an unselected byproduct of selectively adaptive features, which are therefore easily co-opted to a new function.

EXAPTATIONS
Features (such as feathers) that evolved by selection for one purpose (such as warmth) and were later adapted to a new purpose (such as flight).

variation, allowing selection to remodel many different processes at once. In some cases, such as cell-cycle control[13,72–76], HSP90 supports both the activators and the inhibitors of the same function and could uncover the variation that allows selection to either increase or decrease the output of these processes. Moreover, this mechanism is flexible. If HSP90 function is disturbed, developmental pathways are sensitized to a degree determined by their specific dependence on HSP90 (dictated by the functional significance and inherent stability of the relevant targets) and HSP90 availability (dictated in nature by the severity of the stress). Gould argued for the importance of the co-option of structures and mechanisms to new roles during evolution. Similar to his discussion of our inability to predict the future adaptive uses of SPANDRELS and EXAPTATIONS[15], it is impossible to predict whether HSP90-buffered abnormalities *per se* could be adaptive under future conditions. However, relief from HSP90 buffering has a striking ability to generate morphological novelty. Although environmental and genetic robustness can be achieved by known molecular and evolutionary mechanisms, it is more difficult to envision how entrenched developmental systems can be induced to change. The deleterious nature of most mutations does not diminish the importance of rare beneficial alleles for evolution. The possibility of rare beneficial morphologies resulting from polygenic variation that is regulated by HSP90 buffering, and the ability to suddenly produce morphological novelty when the environment changes, might also provide enough of a group selective advantage to drive the evolution of HSP90 buffering.

Selection on a set of targets? The widespread distribution of morphological traits buffered by HSP90 raises the intriguing possibility that the particular network of pathways that rely on HSP90 might have evolved as a set that, if perturbed, provides a bias towards morphological transitions rather than lethality. Many of the proteins that interact with HSP90 in eukaryotes are conserved between humans, plants and other organisms. In the few cases in which it has been examined, HSP90 dependence is also conserved from humans to other organisms: for example, nitrous oxide synthetase in Ascidians[67,68,77], steroid receptors in flies and fungi[63,64,78], MAP kinases and cyclin-dependent kinases in flies[11–13], and a large HSP90 co-chaperone complex conserved from animals to plants and yeast[79–81]. Comparison of HSP90 targets and their sequence-similar, but HSP90-independent, counterparts shows that a small number of amino-acid changes can dictate the difference between HSP90 dependence and independence[35,56]. The genetic and biochemical experiments indicate that any protein might become HSP90 dependent through mutation, and it seems plausible that targets could also evolve HSP90 independence. This raises the interesting possibility that the set of HSP90 targets has been maintained by selection for the group advantage of evolvability that is biased towards exposing variation that affects morphological and other phenotypes that are more likely to produce adaptive variation.

Molecular and evolutionary mechanisms

Gould wrote of evolvability, "phenomena without direct mechanisms generally do not win much interest or approbation from working scientists"[15]. The possible regulation of evolvability is strongly supported by elaborate and well-documented biochemical and molecular mechanisms, although our ability to assess the evolutionary importance of these mechanisms for group selection lags behind our understanding of the molecules involved[82]. Molecular mechanisms for the regulation of genome replication and repair, or for chaperoned protein folding and maintenance, initially evolved on the basis of their clear selective advantages for individuals. However, drastic environmental changes have swept the planet over the history of life. The population and lineage-level benefits of responsively regulating the parameters of evolvability, such as the expressed mutation rate or the generation of developmental and morphological novelty, might one day provide a crucial link in our understanding of biological evolution.

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Acknowledgements
We thank S. Henikoff, S. Rosenberg, W. Fontana and three anonymous reviewers for their insightful comments.

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