Protein Homeostasis and the Phenotypic Manifestation of Genetic Diversity: Principles and Mechanisms

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Abstract

Changing a single nucleotide in a genome can have profound consequences under some conditions, but the same change can have no consequences under others. Indeed, organisms can be surprisingly robust to environmental and genetic perturbations. Yet, the mechanisms underlying such robustness are controversial. Moreover, how they might affect evolutionary change remains enigmatic. Here, we review the recently appreciated central role of protein homeostasis in buffering and potentiating genetic variation and discuss how these processes mediate the critical influence of the environment on the relationship between genotype and phenotype. Deciphering how robustness emerges from biological organization and the mechanisms by which it is overcome in changing environments will lead to a more complete understanding of both fundamental evolutionary processes and diverse human diseases.

INTRODUCTION

"Nothing makes sense in biology except in the light of evolution."

Theodosius Dobzhansky, 1964 (44)

Canalization: insensitivity of a phenotypic trait to mutations or environmental factors

Genetic assimilation: the process through which selection, acting on environmentally contingent traits, renders these traits genetically encoded and stable to environmental change

Natural selection operates on phenotypes rather than genotypes. What is not phenotypically manifest cannot be selected for; therefore, mechanisms that increase the quantity or quality of selectable traits based on underlying genetic variation expand the power of natural selection. Conversely, mechanisms that mask phenotypic variation can limit its influence. The roles of these phenomena in evolutionary processes have been fiercely debated (101, 180).

Empirically, organisms are remarkably resistant to change (5, 12, 39, 50, 63, 76, 87, 157, 176). Extrinsic factors, such as ambient temperature or nutrient availability, change frequently and often unpredictably but generally have little effect on the overt phenotype of an individual. Furthermore, abundant natural genetic variation virtually guarantees that with every generation each gene in an outbreeding sexually reproducing organism finds itself in a genetic environment that has never before existed. Yet, genetic variation too seems to be mostly neutral. Given that natural selection favors traits that are both beneficial and reproducible (122), organisms must have mechanisms that reduce the deleterious effects of such inevitable extrinsic and intrinsic perturbations.

Such mechanisms lie at the core of the interface between genotype and phenotype. However, despite the availability of thousands of fully sequenced genomes, as well as numerous systematic functional studies (43, 62, 74), we still understand frustratingly little about how changes in genotype determine the changes in phenotype on which selection can act (17). This relationship is of fundamental interest to our intellectual understanding of evolutionary processes. It also has profound importance for human health and disease, as well as for our productive and destructive relationships with other species.

Traits that persist despite environmental or genetic perturbations are said to be robust or canalized (**Figure 1**). Canalization has a central role in determining how genotypes are translated into phenotypes, but little is known about the molecular mechanisms that contribute to it. The concept of canalization dates from seminal observations by Conrad Hal Waddington (172, 175) and theoretical work by Ivan Schmalhausen (147). Waddington observed that brief heat shock during fruit fly (*Drosophila melanogaster*) larval development could induce a novel crossveinless wing phenotype in some individual adults in the population (171, 175). Selective interbreeding of the crossveinless flies steadily increased the frequency of the trait in each generation, until it almost reached fixation. Remarkably, after several rounds of selection, heat shock was no longer needed to produce the crossveinless phenotype. The trait had thus become independent of the original environmental stimulus. Waddington argued that significant genetic variation must have preexisted in the original population and that the original phenotype was canalized, preventing its expression. Repeated selection had resulted in genetic assimilation of that variation,

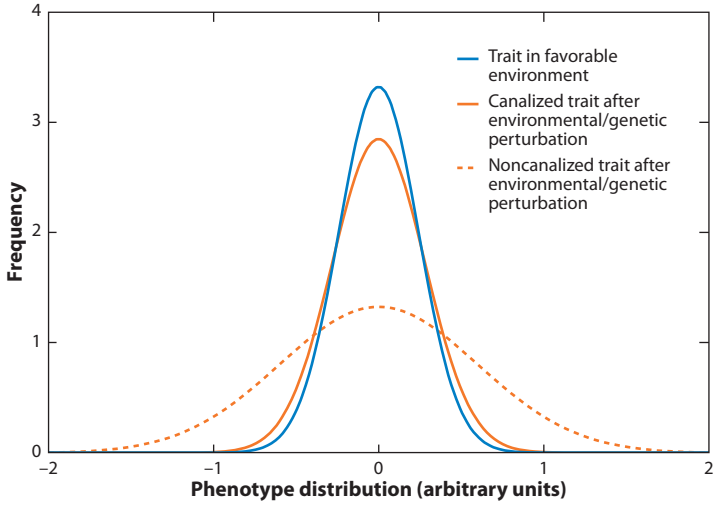


Figure 1

Canalization reduces phenotypic variation. Most traits in nature follow a normal distribution (*solid blue line*). Environmental or genetic perturbations increase the phenotypic variation in noncanalized traits (*dashed orange line*) but have less effect on canalized traits (*solid orange line*).

changing an environmentally induced phenotype to a genetically determined trait (171, 175). However, these results, and the possibility that environmentally induced phenotypes might have a broad role in evolutionary processes, were originally largely dismissed, as they were considered Lamarckian (45).

Our aim here is to highlight the relationship between environmental stress, protein homeostasis, and genotype-to-phenotype transitions because recent discoveries in the field of protein folding are generally outside the purview of evolutionary biologists. We also consider some of the more traditional mechanisms that influence the phenotypic manifestation of genetic diversity, discussing how environmental stress impinges on all of these processes. Finally, we address how canalization can be overcome to enable the evolution of new traits, and we discuss the possibility that buffering capacity and robustness have been selected for over evolutionary time.

MUTATIONS WITHOUT PHENOTYPES?

Perhaps the most surprising result of the genome sequencing projects of the past decade was the realization that complex organisms have many fewer genes than originally predicted (90). Mammals do not have many more genes than invertebrates, which in turn have only two to three times more than the simple unicellular yeast *Saccharomyces cerevisiae*. The inevitable conclusion is that the combinatorial action of relatively few genes is enough to produce the remarkable diversity of life on earth. Another surprise of equal magnitude was that despite the scarcity of genes, many appear to be dispensable. For example, the first genome-wide gene knockout studies in *S. cerevisiae* (62, 153, 187) indicated that over 80% of gene knockouts did not lead to a detectable growth phenotype in a rich medium. Similarly, many homozygous gene knockouts in mice have no apparent phenotypes (11, 25). Haploinsufficiency is even less common (42, 97, 142).

There are two obvious explanations for these findings. First, changes in fitness on the order of 1% would have a significant selective value in natural populations, but are challenging to detect in the laboratory. Again taking up the example of budding yeast, Breslow et al. (22) addressed this issue by measuring fitness consequences of nonessential gene deletions with a highly sensitive competition assay. Almost half of all nonessential gene deletions led to a competition disadvantage in minimal medium, illustrating that many gene deletions do result in subtle but detectable fitness defects. The second explanation is that laboratory conditions inadequately reflect the natural environments in which organisms have evolved over millions of years (128). In a systematic effort, Hillenmeyer et al. (74) subjected each single gene deletion strain in yeast to over a thousand different conditions. They found that 97% of genes are required for optimal growth in at least one condition, demonstrating a condition-specific function for most genes.

MECHANISMS OF BUFFERING AND POTENTIATION

It remains provocative, however, that so many genes in an organism can be deleted without much phenotypic consequence. A diverse cohort of mechanisms has been proposed to contribute to this phenotypic robustness. Here, we highlight the best-characterized mechanisms that play a role in masking or buffering genetic variation. Although we focus on genetic variation, the same mechanisms are likely to apply to the effects of environmental perturbations. Knowledge of mechanisms that enable or potentiate phenotypes associated with new mutations is comparatively fragmentary. We provide examples related to protein homeostasis, but other mechanisms no doubt contribute to the phenomenon.

Diploidy and Duplications

The simplest mechanism for buffering against mutations is increasing ploidy. The vast

Epistasis: nonadditive interaction between genes that control the same phenotype

majority of multicellular organisms are either diploid or polyploid (124). Putting aside the thorny issue of increased mutational load, increased ploidy protects organisms by masking the effects of new mutations. The evolutionary advantage of such masking rests on two fundamental observations. First, many mutations are either deleterious or neutral because organisms are often already well adapted to their environment. Second, most mutations are also recessive. As long as enzymes are not saturated for the substrate and follow Michaelis-Menten kinetics, even simple metabolic pathways can tolerate large changes in individual enzyme activity (83). Increased ploidy would then render such pathways robust to genetic perturbation.

In addition to diploidy, duplications of chromosomal regions or individual genes can buffer against mutations by providing functional redundancy (**Figure 2*a***). Indeed, lack of a homology is a strong predictor of essentiality in *S. cerevisiae*: Only one percent of essential yeast genes have a homolog elsewhere in the genome (paralogs), compared to 8.5% of nonessential genes (62). Nevertheless, it seems surprising that duplications appear to contribute only modestly to robustness. By and large, redundancy appears to be provided by unrelated genes that are part of the same or a compensatory biochemical pathway (66, 70, 78, 176). This is also supported by the most comprehensive systematic profiling study of yeast deletion mutants (74). When a large number of growth conditions are assayed (74), deletions of genes without paralogs are no more likely to result in a phenotype than genes with paralogs.

However, other experiments suggest that selective pressure has acted to retain duplications over long evolutionary periods. Many paralogs in budding yeast arose from a whole genome duplication event approximately 100 mya. In most of these gene pairs, redundant function has been retained over this timeframe, which is long enough for every nucleotide to have been changed assuming even modest mutation rates (40). A similar analysis identified several redundant gene pairs in *Caenorhabditis elegans* that have been maintained since its divergence from *Caenorhabditis briggsiae* approximately 110 mya (160).

Functional Redundancy and Epistasis

Although duplications and diploidy mask variation by offering backup copies for mutated genes, buffering can also result from redundant pathways or interactions between genes (**Figure 2*b***). Perhaps the most striking examples of genetic interactions are those of apparently Mendelian disease mutations that are manifested differently in different individuals, despite identical mutations and similar environmental conditions (112). These observations can be explained by epistasis, or the interaction between an allele and its genetic background. The definition of epistasis varies depending on the context (132). Here, we define it as interaction between genetic loci, i.e., deviation from an additive effect of two or more alleles (133) that is either synergistic (greater than additive effect) or antagonistic (less than additive effect). For example, two mutations show a synergistic epistasis if their combined phenotype is far greater in magnitude than expected from multiplying the individual effects of each mutation in a wild-type background. Likewise, two mutations show an antagonistic epistasis if their combined phenotype is less than would be expected from multiplying the effects of each mutation individually. Antagonistic epistasis generally reveals genes that act in the same pathway, whereas synergistic epistasis generally reveals genes in parallel, or redundant, pathways.

In yeast, the development of systematic arrays of genetic variants to study double mutant or knockdown strains has enabled an unparalleled view of genetic interactions (34, 93, 163, 164). Based on these studies, about 0.5% of genetic interactions between nonessential gene deletions and knockdowns are synthetically sick or lethal (SSL). That is, each nonessential gene in yeast has, on average, more than 30 epistatic interactions. This corresponds to hundreds of thousands of epistatic interactions in the yeast genome, which can be contrasted against

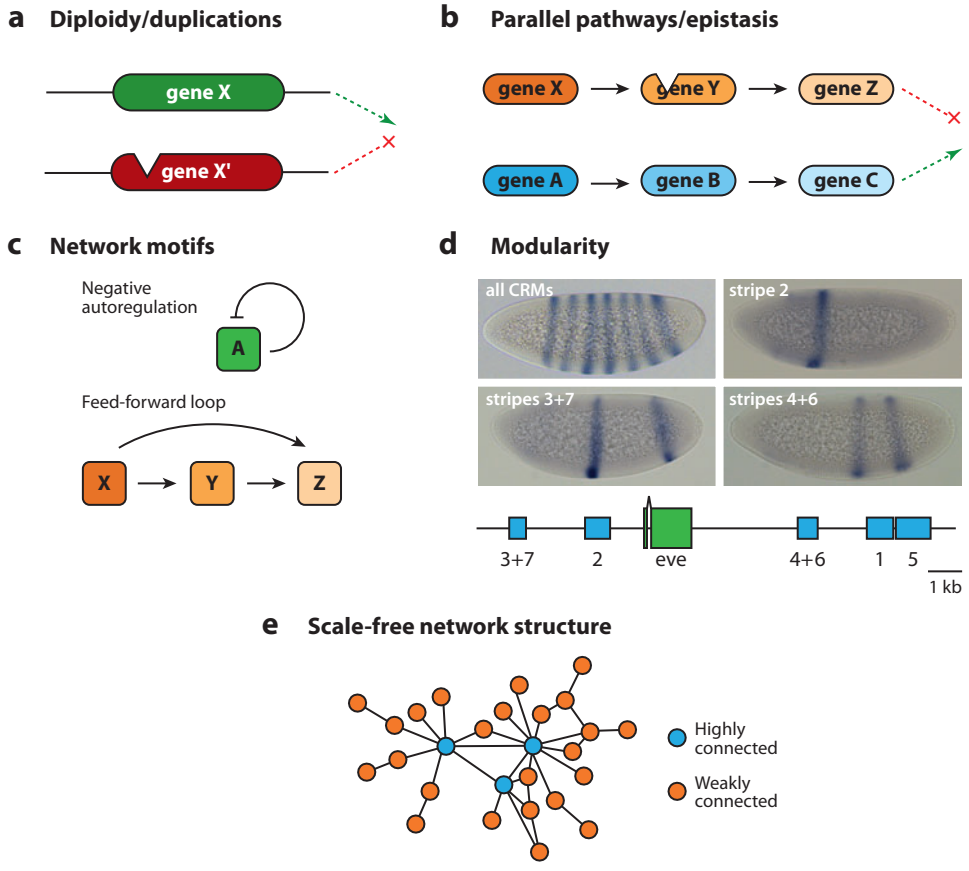


Figure 2

Buffering of genetic and environmental variation is brought about by various mechanisms. (*a*) Diploidy and duplications provide protection from deleterious mutations in one copy of the gene. (*b*) Redundant pathways reduce the effects that perturbation of a single gene has on phenotype. Parallel pathways can be uncovered by epistatic analysis. In this example, mutation in gene Y will have a phenotypic consequence only when cells also carry another mutation in gene A, B, or C. (*c*) (*top*) In negative autoregulation, transcription factor represses its own gene. This circuit reduces variability in gene expression. (*c*) (*bottom*) Transcription factors X, Y, and Z form a feed-forward loop in which both X and Y regulate the expression of Z. Such topology filters out brief fluctuations in the concentrations of X and Y. (*d*) The spatial expression of *even-skipped* gene in *Drosophila* embryo is controlled by five *cis*-regulatory modules (CRMs). Although *even-skipped* gene is expressed in seven stripes, each module directs expression in only one or two stripes. Such modular organization reduces mutational pleiotropy by channeling the effects of mutations. (*e*) Scale-free networks are composed of a few highly connected nodes, or hubs (*blue*), and many weakly-connected nodes (*orange*). Random perturbation of a single node affects the network structure only weakly because most nodes have very few connections. In contrast, directed perturbation of a hub is highly detrimental.

approximately 1,000 genes that are essential (20). These observations illustrate that there is an extensive hidden network of epistasis in the genome. Strikingly similar frequencies have been observed in *C. elegans*. Remarkably, however, the synthetic interactions per se are not well conserved (93, 161). Apparently, the networks connecting conserved protein functions are highly subject to evolutionary change. Across a wide swath of evolution, a

vast network of redundant pathways ensures the robust behavior of biological systems in the face of genetic perturbations and contributes to their ability to evolve new connections.

Networks and Modularity

Response to environmental or genetic perturbations is buffered by redundant genes and pathways, and by the unique manner in which the genome is organized into networks of interacting proteins and functional modules. Several common features of biological organization have emerged from both classical molecular biology experiments and modern high-throughput approaches. Three hallmarks of biological organization that contribute to buffering are motif recurrence, scale-free networks, and modularity.

Biological networks, such as transcription regulation networks and signal transduction pathways, are enriched in recurrent building blocks termed motifs. Strikingly, the motifs that are enriched in biological systems tend to increase stability (135). For example, as revealed by studies in both bacteria and yeast, two distinct motifs are overrepresented in transcriptional networks: negative autoregulatory loops and feed-forward loops (FFL). An example of negative autoregulation would be a transcription factor that binds its own promoter and represses its own expression once it has accumulated to a sufficient level to bind its other target sites (4, 107) (**Figure 2*c***). This simple motif confers stability to gene expression levels and reduces the effect of extrinsic or intrinsic perturbations (15). In contrast, FFLs are composed of three components: X regulates downstream components Y and Z, whereas Y regulates only Z (**Figure 2*c***). Coherent FFLs, in which all components are transcriptional activators, stabilize gene expression by filtering out the effects of brief fluctuations in the concentration of upstream regulators (4).

Biological networks are typically composed of independently functioning modules that are only weakly connected to each other (72, 178). Such organization channels the effects of mutations by reducing pleiotropy and allowing traits to evolve more independently. For example, temporal or tissue-specific gene expression patterns are often controlled by independent transcriptional enhancer sequences called *cis*-regulatory modules (CRM). Thus, the pair-rule gene *even-skipped* is expressed in seven stripes in the developing *Drosophila* embryo. Its expression is controlled by five independent CRMs, each driving expression in one or two stripes (**Figure 2*d***). The consequences of mutation in any one module are limited to that stripe. Modularity of gene regulatory networks has been proposed to be a major force in the evolution of novelty (27), although this view has also been vigorously contested (75, 101).

On a larger scale, much of the observed robustness of biological networks results from their highly nonuniform scale-free structure (2). Some nodes serve as highly connected hubs with many interactions, but most nodes have only a few connections (10) (**Figure 2*e***). Consequently, the network is highly resistant to perturbations, given that the vast majority of nodes are not centrally connected (2). A remarkable example of network robustness comes from studies in *Escherichia coli*. Isalan et al. (80) rewired transcriptional networks by randomly connecting transcription factors to inappropriate promoters and studied the outcome of nearly 600 such circuits over a wild-type background. Wild-type networks tolerated more than 95% of these constructs (at least under laboratory growth conditions), illustrating the robustness of the networks (80). However, the tradeoff of scale-free networks is that they are vulnerable to targeted perturbations of well-connected hubs (2). Siegal & Levy (95) measured the effects of all nonessential gene deletions on morphological variation in budding yeast. They discovered more than 300 genes that contribute to buffering of morphological variation. Significantly, these gene products appeared to be enriched in network hubs with a high number of protein-protein interactions and genetic interactions (95).

PROTEIN FOLDING AND CHAPERONES

In addition to the aforementioned mechanisms, an emerging body of evidence suggests that protein homeostasis profoundly influences the relationship between genotype and phenotype. The functioning of protein homeostasis networks acutely depends on the environment. In turn, the master regulators of the heat-shock response (the transcriptional regulator HSF1 in eukaryotes and sigma factors in prokaryotes) are repressed by the very chaperones whose expression they induce. Environmental stresses recruit these chaperones to newly unfolded proteins, liberating the transcriptional regulators to activate the heat-shock response until problems in protein homeostasis have been resolved. This feedback mechanism buffers protein homeostasis against moderate environmental perturbations. However, with greater extremes of environmental fluctuation, the intrinsic dependency of protein homeostasis on the environment allows it to exert particularly powerful effects on the evolution of new traits.

To function, proteins must adopt complex three-dimensional conformations in a challenging intracellular environment where macromolecular crowding can derail even the most robust biological pathways (8, 52). The concentration of proteins in living cells is astonishingly high, 300 mg ml^{-1} (52). Moreover, many proteins are inherently unstable. In vivo, folded, native conformations are usually maintained with free energy of unfolding (ΔG) of about –5 to –15 kcal mol^{-1} (59), which roughly corresponds to the free energy of hydrolysis for a single molecule of ATP. Even minor perturbations can convert active conformations into unfolded, inactive, and often toxic states (59). Moreover, protein aggregation itself has considerable sequence-independent toxic effects (24). Although the tolerance of proteins to missense mutations varies greatly, most point mutations alter protein stability ($\Delta\Delta G$) by 0.5–5 kcal mol^{-1} (41). Protein function can be negatively affected by mutations that decrease stability even if they do not affect catalytic activity or substrate specificity. The effect is amplified by the susceptibility of unfolded proteins to degradation. Protein stability is therefore a major constraint on the evolution of new functions (19). Indeed, because mutations in the hydrophobic core of a protein tend to be more destabilizing than those on the surface, such changes are less tolerated. More broadly, the central role of protein homeostasis in normal biology is underscored by the many different human pathologies that arise from defects in protein folding (8).

Chaperone: molecule that promotes protein folding or complex assembly but does not otherwise contribute to function

To contend with problems inherent to protein folding, all organisms employ an elaborate repertoire of homeostatic mechanisms (191). These include the synthesis of osmolytes, protein chaperones, and protein remodeling functions. Collectively, these agents usher newly synthesized proteins into active conformations and promote the refolding of proteins that have lost their normal conformations in the highly dynamic cellular milieu. Environmental stresses can disrupt this equilibrium, creating a crisis in protein homeostasis. Cells respond by rapidly inducing a subset of these chaperones and remodeling factors, collectively known as heat shock proteins (Hsps) (96). By enabling the folding and therefore function of a wide variety of proteins that would otherwise misfold and aggregate, chaperones have a central role in protecting organisms from extrinsic perturbations. Recent findings have led to the compelling hypothesis that protein folding machinery can buffer and potentiate genetic variation.

HSP90

The Hsp90 chaperone is conserved from bacteria to humans. Yet, although Hsp90 (*htpG*) is dispensable in bacteria, it is essential in every eukaryote that has been tested. Hsp90 is extremely abundant—constituting ~1% of total protein under normal growth conditions—and these levels are increased approximately twofold by environmental stress (21). It functions in virtually all compartments of the eukaryotic cell: Hsp90 paralogs are present in

the endoplasmic reticulum, mitochondria, and even chloroplasts (29).

Among chaperones, Hsp90 is unusual in at least two respects. First, although very high levels of the protein are needed under conditions of protein homeostatic stress, the levels normally present are much higher than required for growth. This excess Hsp90 provides a large reservoir of chaperone capacity under normal conditions.

Second, Hsp90 substrate proteins (commonly referred to as clients) are enriched in key biological regulators (144). The metastable conformations of such proteins are intrinsic to their function as signal transducers. For example, Hsp90 associates with steroid hormone receptors in the cytoplasm and stabilizes them in the absence of their ligands, maintaining them in a state that is competent for binding (134, 152). Similarly, Hsp90 is required for the maturation and/or stability of a large number of kinases (30). Genome-wide functional assays in yeast have revealed that Hsp90 physically or genetically interacts with a very diverse set of clients involved in pivotal cellular processes, including signal transduction, chromatin remodeling, protein trafficking, and the cell cycle (104, 106, 194).

These observations, together with Hsp90's strong effects on the phenotypic manifestation of genetic variation (discussed below), suggest that the greatly increased importance of Hsp90 in eukaryotes compared to prokaryotes may have played a role in the evolution of novelty in this kingdom. Many important pieces of the puzzle are still missing. Yet, several lines of evidence suggest a framework for understanding the effects that Hsp90 can have on the traits conferred by genetic variation. It acts in at least two ways.

First, it can buffer the effects of variation. Under normal growth conditions, the reservoir of Hsp90 chaperone activity keeps signaling pathways functioning optimally, allowing variation to accumulate without phenotypic consequence. However, when environmental stress compromises the Hsp90 reservoir, the effects of previously hidden genetic variation are released in a combinatorial fashion. This provides a means for producing genetically complex traits in a single step.

Second, the Hsp90 reservoir can potentiate the effects of variation, allowing it to have immediate phenotypic consequence. Variants that are unstable or that require Hsp90 clients to persist can thereby exert new functions, providing a means for the rapid evolution of new traits. In this case, when the Hsp90 reservoir is compromised by environmental stress, the traits encoded by these variants disappear.

Both of these mechanisms can operate simultaneously in the same individual, and importantly, traits affected by either mechanism can be genetically assimilated via selection. In the case of Hsp90-buffered traits, this can occur via reassortment of the underlying variation. In the case of Hsp90-potentiated traits, it can proceed via the acquisition of new mutations that are robust to environmental perturbation. In each case, the interface between the reservoir of Hsp90 function and the environment provides a powerful means of transforming the adaptive value of variation (see below).

Buffering of Genetic Variation

Results from a range of eukaryotic model organisms have established that Hsp90 can act as a pervasive buffer against the effects of genetic variation. Genetic or pharmacological impairment of Hsp90 function caused a profusion of new morphologies that affected diverse adult structures in *Drosophila* (141) (**Figure 3*a***). Given Hsp90's role in the folding of key developmental regulators, it was not terribly surprising that reducing Hsp90 function would destabilize development. What was surprising was that completely different phenotypes were produced in different flies. These were reproducible and strongly depended upon the genetic background. A commonly held belief in evolutionary theory is that genetic polymorphisms affecting important developmental traits are not allowed to accumulate. This work revealed that such polymorphisms are, in fact, common, but their effects are generally

masked by the chaperone activities of Hsp90. Consistent with a pivotal role in ensuring uniformity of phenotype, traits affected by Hsp90 inhibition are among the most canalized in *D. melanogaster* (108). Importantly, such traits can be revealed by a physiologically relevant temperature shift, suggesting a common avenue through which buffered traits could be revealed and selected upon in nature. Indeed, selection can lead to genetic assimilation of these traits.

The role of Hsp90 in canalizing development under normal circumstances and the effects of protein folding stress in releasing canalization are not oddities of fruit flies. In *Arabidopsis thaliana*, an inbreeding organism with far less heterozygosity than *Drosophila*, reducing Hsp90 function affects many morphological features of the plant, including cotyledons, hypocotyls, root morphology, rosettes, and pigmentation (143) (**Figure 3*b–d***). Moreover, Hsp90 affects a wide variety of life history traits, including hypocotyl extension in the dark and flowering time.

The precise phenotypes again depended on genetic background. A physiological temperature shift from 22°C to 27°C induced a comparable response, indicating that subtle environmental changes are sufficient to elicit dramatic Hsp90-dependent alterations in phenotype. A similar phenomena, though less extensively characterized, has also been reported in zebrafish (190) (**Figure 3*e***). Hsp90 thus contributes to canalization of development, and its inhibition can dramatically alter the effects of underlying variation.

Genetic crosses in both *D. melanogaster* and *A. thaliana* clearly demonstrated that many phenotypic alterations revealed by Hsp90 inhibition are genetically determined. Furthermore, many Hsp90-dependent life history traits could be mapped to quantitative trait loci in *Arabidopsis* (145). Critically, however, the precise genetic changes involved remain to be elucidated and their adaptive value is yet to be demonstrated.

Other work indicates that some Hsp90-buffered phenotypes in *Drosophila* are epigenetically determined (140, 155). In addition, an exciting link to transposon control by Hsp90 was recently discovered, and this too can lead to the appearance of novel and reproducible phenotypes when Hsp90 function is compromised (156). Here again, the mechanisms are not yet well understood. Hsp90 client proteins include several chromatin proteins (194) and Ago1, a protein involved in small RNA processing (158), which are plausible candidates for further study. Hsp90 function is also important for mutagenesis induced by DNA damage in human cell lines (148).

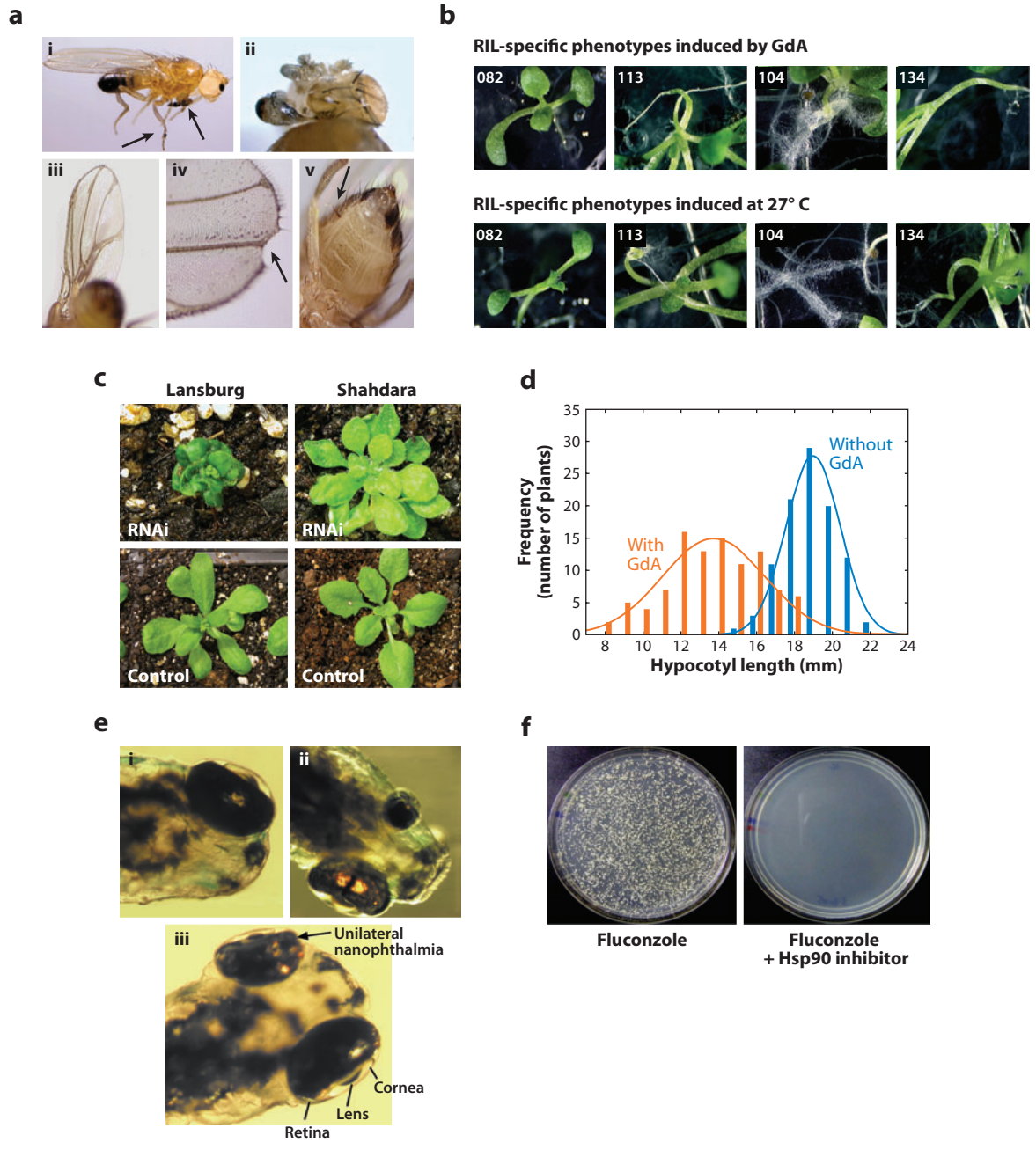
Hsp90 Potentiates Genetic Variation by Directly Stabilizing Metastable Proteins

In addition to buffering variation, Hsp90 can potentiate the ability of new variants to create immediate new phenotypes. Most mutations, especially those that confer new functions, also destabilize protein structure, sometimes enough to prohibit the acquisition of a functional fold. Hsp90 can bind such variants and rescue their folding or stability defects. In such cases, instead of hiding the effects of accumulating genetic variation, the reservoir of Hsp90 chaperone activity enables new functions that would otherwise be hidden. Currently, Hsp90-dependent oncogenic mutations provide the only clear examples. The first identified was the promiscuously activated tyrosine kinase v-Src (183, 188). Whereas its normal counterpart (c-Src) only transiently interacts with Hsp90 (189), oncogenic v-Src forms a stable complex (121). V-Src lacks a C-terminal regulatory residue present in c-Src (33) and is much less stable than c-Src (54). c-Src function is only modestly dependent upon Hsp90, but it is only with the help of Hsp90 that v-Src can orchestrate oncogenic transformation (54, 183).

Hsp90-dependency has now emerged as a common feature of many oncogenic mutations. Oncogenic alleles of a wide range of kinases, including BRAF, EGFR, ErbB2, FLT3, LCK, and the translocation fusion gene Bcr-Abl, exhibit Hsp90-dependent activities (182). In this light, it is striking that Hsp90 is preferentially found as a multiprotein complex in tumor cells,

given that it generally is in a latent, noncomplexed state in normal cells (84), although this important result needs to be confirmed. Hsp90 is a promising target for cancer therapy, and several Hsp90 inhibitors are being tested in clinical trials (99, 182).

Hsp90's ability to potentiate genetic variation can also be indirect. The only molecularly understood example comes from Hsp90-dependent resistance to azole antifungal therapeutics in yeast. Strains with reduced Hsp90 levels acquire resistance to fluconazole at a



much lower frequency than those with a normal reservoir of Hsp90 function (36) (**Figure 3*f***). In *S. cerevisiae*, most such mutations obtained in laboratory selections are in *ERG3*, rather than the azole target protein Erg11. However, because Erg3 is important for membrane biogenesis, these mutations also have cytotoxic secondary effects. Cytotoxicity is counteracted by Calcineurin, an unstable Hsp90-dependent phosphatase that coordinates several cellular responses to azoles and membrane stress (36, 38, 61, 79). Reducing Hsp90 function prevents Calcineurin from properly functioning and renders the resistant strains susceptible to the full collateral damage of *ERG3* mutations. Hsp90's influence on azole resistance extends beyond mutations in *ERG3*. A genome-wide screen identified 11 deletion mutations that confer fluconazole resistance (6). Hsp90 was required for resistance in each case (36). Pathogenic fungi with fluconazole resistance that was acquired in a human host provided a means to test whether natural evolution of drug resistance also depends on Hsp90. It does in each of several cases that have been examined. Moreover azole resistance, once acquired, continues to depend on the reservoir of Hsp90 chaperone function (36).

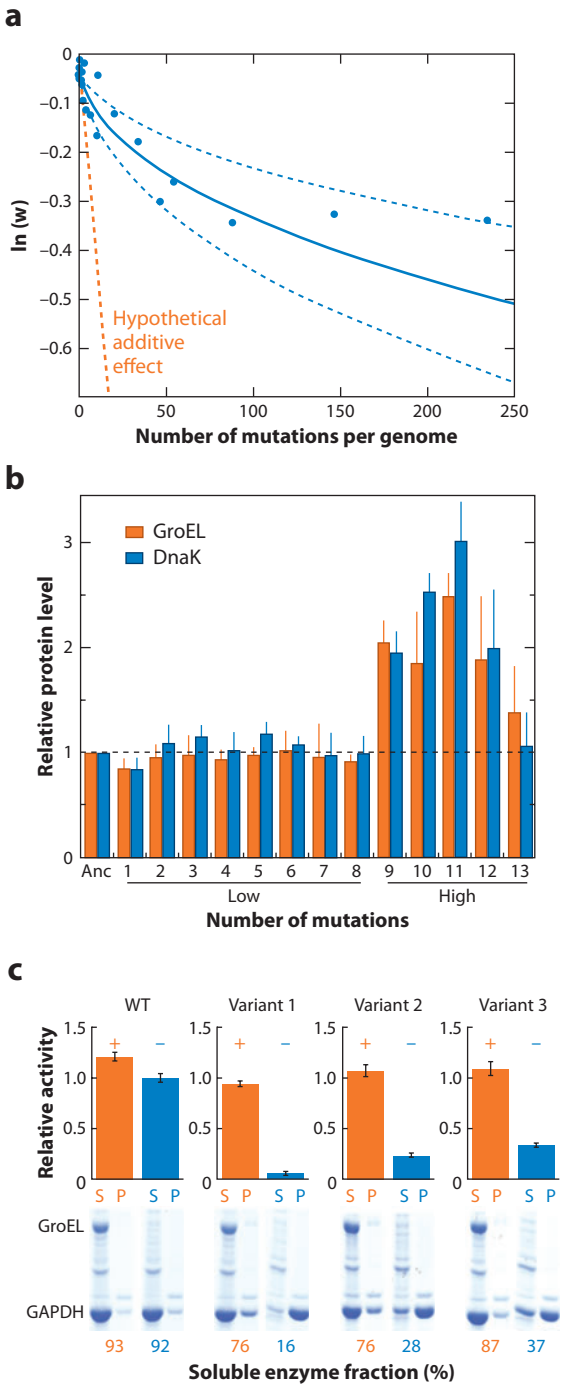
The broad outline of these mechanisms for evolving resistance to antifungal agents is conserved in human pathogens separated by nearly 1 billion years of evolution (73). Hsp90 potentiates resistance to azoles in *Candida albicans* and to a completely different class of antifungals, echinocandins, in clinical isolates of *Aspergillus terreus* (36). In both organisms, Hsp90's effects on drug resistance arise through mechanisms that are strongly dependent on Hsp90, and at least part of this effect is exerted through Calcineurin (36). In principle, a similar schema could underlie the potentiation of new traits that depend on any Hsp90 client. Notably, Hsp90-potentiated traits are also affected by changes in the environment, but in the opposite direction as Hsp90-buffered traits (see below).

GroEL BUFFERS AND POTENTIATES GENETIC VARIATION IN BACTERIA

Although the bacterial Hsp90 homolog (HtpG) has not been reported to buffer or potentiate genetic variation, an emerging body of evidence suggests that another chaperone, GroEL, may fulfill this function. GroEL belongs to the chaperonin family of chaperones and facilitates the folding of many client proteins through successive cycles of binding, encapsulation, and release (71). It does so by forming a cage that binds substrates on the rim of its interior chamber. GroES, another chaperonin encoded in an operon with *groEL*, functions as a lid, closing over the chamber. Hints to the possibility that GroEL could buffer genetic variation came from early studies showing that its overexpression could suppress the phenotypes of many

Figure 3

Hsp90 buffers and potentiates genetic variation in a variety of model organisms. (*a*) In *Drosophila*, a small fraction of heterozygous *Hsp83/+* individuals OK show a range of morphological abnormalities that depend on underlying genotype. (*i*) Deformed fore-leg and transformed second leg (L2) with ectopic sex-comb (*arrow*); (*ii*) deformed eye with an extra antenna; (*iii*) thickened wing veins; (*iv*) notched wings; (*v*) extraneous tissue growing out of tracheal pit. (*b*) Phenotypes induced in specific recombinant inbred lines (RIL) of *Arabidopsis thaliana* by treatment with the Hsp90 inhibitor geldanamycin (GdA) and recapitulated with temperature stress. Line 082 seedlings show S-shaped rosettes with vertically oriented leaf blades. Line 113 seedlings show extreme hypocotyl curls and roots partially extended into air. Line 104 seedlings show abundant root hair growth. 134 seedlings show bent hypocotyls with rosette touching the medium surface. (*c*) Reduction of Hsp90 levels by RNA interference in Landsberg erecta and Shahdara ecotypes reveals leaf malformation and overproliferation, respectively. (*d*) Histogram of hypocotyl length in the dark from RIL growth with (*orange*) or without (*blue*) GdA reveals both a broadened distribution and shift in median phenotype upon Hsp90 inhibition. (*e*) Morphological abnormalities associated with the Hsp90-revealed trait in zebrafish: (*i*) unilateral anophthalmia; (*ii*) unilateral microphthalmia; (*iii*) unilateral nanophthalmia, illustrating retina, lens, cornea. (*f*) Resistance of *Saccharomyces cerevisiae* to 128 μg/ml fluconazole is abolished by coadministration of the Hsp90 inhibitor radicicol.



unrelated temperature-sensitive alleles in both bacteria and phage (58, 81, 115, 168). Bona fide support came from later mutation accumulation studies in *Salmonella typhimurium* and *E. coli* (57, 102). Fares et al. (57) compared fitness of *E. coli* strains subject to 10,000 generations of adaptation to a new growth condition. In the process, the evolved strains lost considerable fitness in ancestral growth conditions. However, increased GroEL expression rescued much of this decline (57).

In a complementary study, lineages of *S. typhimurium* with widely varying mutation rates were created from a single parental strain. As expected, fitness declined with increasing mutation load, but the relationship was strikingly nonlinear. Indeed, fitness loss eventually reached a plateau with increasing numbers of mutations per genome (102) (**Figure 4*a***). How can this plateau be explained? An inconceivably high rate of compensatory mutation would be required for simple widespread epistasis to account for it (102). However, GroEL levels were elevated in lineages with the highest numbers of mutations (**Figure 4*b***), and further deliberate GroEL overexpression rescued much of the fitness decline present in the highly mutated lineages but did not compromise the

Figure 4

Chaperones buffer and potentiate genetic variation in bacteria. (*a*) Plot of log relative fitness versus number of mutations per genome in *Salmonella typhimurium* lineages generated over 8,000 generations with varying numbers of mutations per genome (*blue*). Confidence intervals (95%) are plotted in dotted lines around the fit. The hypothetical trend line represents the expected relationship for an additive effect for each mutation (*orange*). (*b*) Plot of GroEL (*orange*) and DnaK (*blue*) levels relative to the ancestral strain (Anc) in lineages passaged with low and high numbers of mutations shows that each of those with the greatest mutation load overproduce GroEL. (*c*) GroEL expression affects stability of new variants in *E. coli*. WT glyceraldehyde-3-phosphate dehydrogenase depends on GroEL chaperone function for activity (orange bars with GroEL overexpression versus blue bars without excess GroEL), and variants from selection experiments do even more so. Abbreviations: P, pellet; S, soluble.

parental strain (102). Collectively, these findings indicate that GroEL can buffer the destabilizing effects of mutations on the bacterial proteome.

GroEL-dependent buffering may also be crucial to the survival of endosymbiotic bacteria. Many eukaryotes, particularly insects, complement their metabolic deficiencies through interaction with obligate endosymbionts (14). Theoretically, such endosymbiotic bacteria risk fixation of deleterious mutations due to genetic drift (56, 110). Yet, many endosymbioses are long-lived. As just one example, the symbiosis between *Buchnera*, a relative of *E. coli*, and termites has persisted for 100 million years (16, 151). A factor that buffers deleterious mutations is therefore likely to exist in endosymbionts. GroEL is an attractive candidate because its expression is very high in certain endosymbionts (up to 10% of total protein) (57, 110, 146). Additional evidence for GroEL in buffering genetic variation comes from analysis of *groELS* operon, indicating that it has been subjected primarily to purifying selection (55). In endosymbionts from distant phyla, positively selected mutations occur at the same amino acid positions in GroEL and GroES. These amino acids govern GroEL binding to GroES and interaction with unstable client proteins (55, 56). A plausible interpretation is that this positive selection may reflect a metamorphosis of GroEL from a transient responder to environmental stress in free-living bacteria to a constitutive guardian of fitness in the face of genetic drift in endosymbionts (56).

These properties of GroEL have been harnessed to facilitate directed evolution, providing further insights into its function. Tokuriki & Tawfik (162) subjected several GroEL clients to random mutagenesis, simulating genetic drift. In selection experiments with the client glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GroEL overexpression led to a far greater number of variants. This is presumably because excess GroEL potentiated many variants by allowing them to acquire functional folds. Furthermore, the variants were several-fold more active on average. This may be because mutations that change enzymatic activities are frequently located in the hydrophobic core and are more destabilizing than surface mutations. They are therefore more likely to depend on GroEL for stability. Indeed, GAPDH variants recovered from selection with GroEL overexpression were, on average, 2.5 kcal mol^{-1} less stable than those recovered from selections with normal GroEL activity. This phenomenon might be restricted to GroEL clients; control selections with a nonclient (triose phosphate isomerase) showed very little dependency on chaperone function. Collectively, these findings provide evidence that GroEL-mediated buffering and potentiation can sculpt adaptive landscapes in bacteria and pioneer the application of this framework to diverse problems of medical and technological importance.

FUNGAL PRIONS

The yeast prion *[PSI+]* provides another molecular mechanism for the storage and release of cryptic variation. Prions are self-perpetuating protein conformations that have altered functions and, critically, can convert the nonprion conformation into the prion state (150). Because proteins with the altered prion conformation are passed from mother cells to their daughters, they function as protein-based elements of inheritance.

One of the best-characterized prions is formed by the translation termination factor Sup35 in fungi. In its nonprion form, Sup35 ensures faithful translation termination. However, in a small fraction of genetically identical cells, Sup35 spontaneously adopts a self-perpetuating, nonfunctional aggregated conformation. This self-templating conformation creates the heritable genetic element known as *[PSI+]*. *[PSI+]* titrates away soluble and functional Sup35, increasing the readthrough of stop codons and the rate of ribosomal frameshifting (116, 165, 166). This can result in translation of sequences downstream of stop codons (leading to alternative C-terminal sequences in proteins) or changes

in mRNA stability, among other effects on the proteome (150).

The *[PSI+]* state produces a wide range of phenotypes that strictly depend on the genetic background. This is because sequences downstream of stop codons are under reduced selective pressure and can vary greatly. A substantial fraction of traits produced by *[PSI$^+$]* are clearly beneficial under particular growth conditions and their genetic architecture is frequently complex (165, 166). Furthermore, *[PSI$^+$]*-dependent traits could become genetically assimilated via new mutations (discussed below). Together, these observations suggest that *[PSI+]* acts on cryptic genetic variation to promote survival in fluctuating environments and could also facilitate the rapid evolution of complex traits.

The idea that *[PSI+]* could serve as an evolutionary capacitor remains controversial (23, 101, 114, 129, 154). Indeed, it is clear that the *[PSI+]* state is generally slightly deleterious; if *[PSI+]* was unconditionally beneficial, it would have been rapidly fixed by selection. However, Sup35p has retained its ability to convert to *[PSI+]* for over 800 million years of evolution, suggesting an important intermittent function for prion formation. Population genetic models with realistic assumptions have shown that the ability to switch to *[PSI+]* could have been maintained in yeast populations due to its ability to generate adaptive variation during stress (103). Importantly, a wide variety of stressful conditions increases the rate at which cells convert to *[PSI$^+$]* (167). Thus, when an organism is not well suited to its environment, problems in protein homeostasis make it more likely that the self-perpetuating prion aggregate will form. This acts upon previously hidden genetic variation to create novel phenotypes. If even only a fraction of these are advantageous under the cirumstance, *[PSI$^+$]* can confer adaptive value to those cells that switch.

The recent discovery that the yeast genome encodes a surprising number of proteins capable of forming prions further points to the intriguing possibility that they may broadly serve as a bet-hedging mechanism (3, 49, 118, 130). Alberti et al. generated and employed an algorithm to identify proteins across the yeast genome with domains that would be capable of adopting heritable self-templating aggregated conformations (3). An astonishing number of these domains (25) functioned as prions in vivo.

Most strikingly, these new prion domains were strongly enriched in proteins that affect information flow, such as transcription factors and RNA binding proteins. This observation hints at the possibility that many of these new prions may function as phenotypic capacitors. One new prion examined in depth, *[MOT3+]*, exemplified the potential of these new candidate prions to function at the interface between genetic and phenotypic diversity. The causal agent of *[MOT3+]* is a transcription factor, and the prion exerts broad influence on the expression of many genes. Furthermore, *[MOT3+]* arises naturally at a high frequency (one in 10,000 cells). Indeed, the frequency at which many prions appear may be tuned to optimize the phenotypic heterogeneity they provide (69).

ENVIRONMENTAL STRESS AND VARIATION

If traits are resistant to environmental and genetic perturbations, how do organisms adapt to new environments and ultimately evolve? Canalized traits, by definition, are tolerant to genetic variation. Thus, significant variation can accumulate in populations under stabilizing selection (91). In an extreme case, canalization might lead to an evolutionary lock-in, rendering organisms unable to adapt, despite a normal supply of mutations (94). However, in many cases robustness can, counterintuitively, facilitate the acquisition of novelty (46).

This paradox can be explained by neutral networks, which describe the connected set of genotypes that share the same phenotype (177). Mutationally robust traits have large neutral networks, which allows organisms to populate a wider spectrum of genotypes while maintaining a constant phenotype (19, 46, 77, 98, 177). The set of phenotypes that can be accessed by further mutation, i.e., the phenotypic

Neutral network: the set of mutations that can be acquired by a given genotype without affecting phenotype

neighborhood, can therefore be larger for robust traits (**Figure 5**). Environmental changes that perturb the protein homeostasis can have profound effects on such neutral networks. On the one hand, environmental perturbation of buffering can lead to a smaller neutral network due to phenotypic exposure of previously neutral genotypes. These phenotypes can be either deleterious (white circles in **Figure 5*b***) or further adaptive (green population in **Figure 5*b***). The phenotypes uncovered by Hsp90 compromise in flies and plants are experimental examples of such changes. On the other hand, distinct genotypes can become phenotypically similar upon loss of buffering (cyan population in **Figure 5*b***). One such example is the loss of azole resistance in fungi upon inhibition of Hsp90 function.

Thus, the amount of genetic variation available for natural selection to act upon is modulated in response to environmental stress. A variety of processes contribute to this phenomenon. Two broadly important mechanisms are the ability of environmental stress to induce new genetic variation and to affect preexisting mutations by compromising mechanisms that normally buffer or potentiate them.

Induction of New Genetic Variation

It is broadly appreciated that environmental stress can directly affect the rate at which

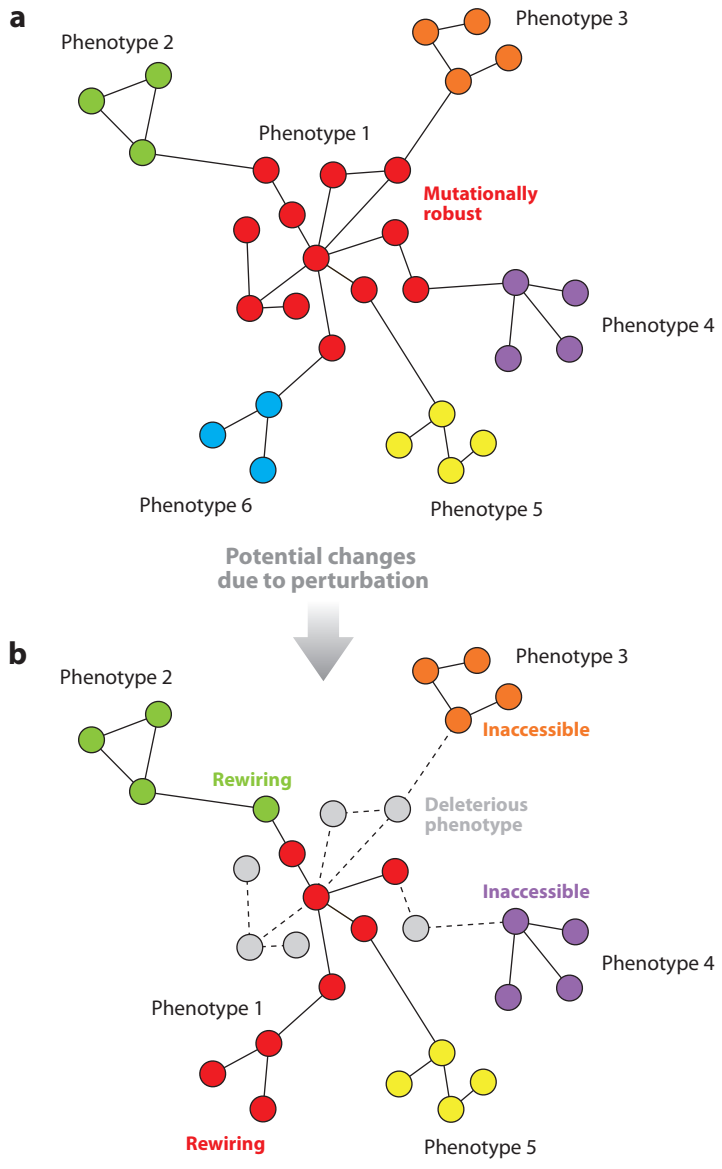


Figure 5

Neutral networks, mutational robustness, and adaptation. (*a*) Neutral networks consist of genotypes (*circles*) connected by single mutations (*lines*) that have the same phenotype (*colors*). A mutationally robust trait (*red*) has a large neutral network and access to multiple neighboring phenotypes through subsequent mutations. For clarity, deleterious mutations are not shown. (*b*) Perturbation of buffering mechanisms influences neutral networks in several ways. Loss of buffering can lead to a smaller neutral network and phenotypic neighborhood, as many genotypes become phenotypically deleterious (*gray circles and dashed lines*). Thus, phenotypes can become inaccessible (*orange*, *purple phenotypes*). Perturbation can also lead to a rewired genotype-to-phenotype map [*green and cyan phenotypes from* (*a*)]. For example, in Waddington's original assimilation experiment, phenotypically identical flies exhibited different phenotypes upon heat shock [*change from red in* (*a*) *to green in* (*b*)]. However, the effects can also be the opposite; distinct phenotypes become identical despite the genotypic differences [*change from cyan in* (*a*) *red in* (*b*)]. An example of the latter is the observation that inhibition of Hsp90 function can revert the cellular transformation orchestrated by an oncogenic kinase, v-src.

Proteostasis: proteome-wide maintenance of protein folding, concentration, interactions, and localization

variation is created. Perhaps the best-established environmentally responsive mechanisms for increasing genotypic diversity are recombination and sexual reproduction. Both sex and recombination increase variation by bringing together new combinations of alleles. They also provide an opportunity to purge deleterious mutations from the population (67). Most molecular biologists are familiar with the protocol to induce sexual reproduction in budding or fission yeasts: starve the cells in a nutrient-poor medium. Stress-induced recombination and sex have also been well documented in a wide range of multicellular organisms (60, 89, 92, 117, 169, 179). Similarly, bacteria can become competent to take up foreign DNA from the environment and to exchange DNA with each other as a response to environmental stress or DNA damage (31).

Together, these observations have led to an "abandon ship" model of recombination and sexual reproduction (1, 68). Increased recombination and sex in unfit individuals would facilitate the escape of beneficial alleles from an unfavorable genetic background and increase their chances of finding a more compatible genetic context (1, 68). Such fitness-associated sex could even explain some aspects of the evolution of sex, which is perhaps the most enigmatic question in evolutionary biology (68).

Environmental stress can also directly affect mutation rate. In bacteria, many stresses increase mutation rate by inducing the SOS transcriptional response and other mutagenic mechanisms (18). Many bacteria and yeast also harbor contingency loci, which are hypervariable regions in the genome that regulate host-pathogen interactions (111, 138, 170). It has been proposed that stress-induced activation of transposable elements may play a role in adaptation (105). Although this idea has been controversial, studies in rice indicate that alteration in flowering time by transposon insertion may have played an important role in domestication of temperate cultivars (82, 113). Intriguingly, a recent study also suggests that the Hsp90 reservoir plays a critical role in suppressing transposition in *D. melanogaster* (156).

Protein Homeostasis and Phenotypic Variation

It is well established that extrinsic factors directly influence and easily perturb cellular protein homeostasis. Chaperones and other mechanisms maintaining proteostasis are thus uniquely positioned at the interface between the environment and genotype-to-phenotype transition. On the one hand, chaperones such as Hsp90 unequivocally contribute to robustness by buffering genetic variation (57, 137, 141). On the other, protein homeostasis mechanisms provide an attractive molecular mechanism by which the accumulated variation can be revealed during environmental stress, which might help organisms to adapt to novel environments. Although chaperone activity is upregulated under stressful conditions (e.g., elevated temperatures), this increase is sometimes insufficient to fully cope with ensuing destabilization of the proteome. Such environmental stresses would therefore provide a common means for compromising protein homeostasis-related buffering systems. Although this hypothesis remains to be fully investigated, it is strongly suggestive that temperature stress can recapitulate phenotypes revealed by changes in Hsp90 function in *Drosophila*, *Arabidopsis*, *S. cerevisiae*, and *C. albicans* (36, 137, 141).

Environmental stress can also exert strong effects on traits that depend upon *[PSI$^+$]*. Tyedmers et al. performed an unbiased screen for genes that influence the induction of *[PSI+]* and found a significant enrichment for genes involved in stress responses (167). Further, they screened environmental conditions that induced *[PSI+]* and determined that diverse environmental stresses increase the switching rate to *[PSI+]* up to two orders of magnitude (167). The extent of these increases was proportional to the severity of the stress. These findings strongly support the notion that *[PSI+]* constitutes an epigenetic bet-hedging mechanism by which genetically identical cells within a population can convert previously latent phenotypic variation into heritable new phenotypes in response to stressful conditions (167).

Genetic Assimilation of Environmentally Contingent Traits

The environmental dependency of many buffered and potentiated traits means that even those that are advantageous constantly risk eradication. What mechanisms might allow such traits to escape environmental contingency and become genetically assimilated? After repeated selective breeding, the morphologies that Waddington first observed in response to stress in *Drosophila* were expressed even in the absence of stress (171, 173–175). This led him to propose that separate mechanisms would exist to enable environmentally acquired traits and to facilitate their genetic assimilation. Half a century later Hsp90 offers an attractive unifying molecular mechanism for these findings (141). In *D. melanogaster*, rare traits (present in approximately 1% of the population), which were initially dependent on genetic reduction of Hsp90 function, could be enriched by selective breeding until they were eventually expressed in 80–100% of the population. However, not a single fly (of more than 50 examined) retained the initial Hsp90 mutation at the conclusion of the selection (141). These traits therefore lost Hsp90-dependency during selection. Just as in Waddington's experiments (171, 173–175), the combinatorial effects of multiple environmentally sensitive polymorphisms were responsible for genetic assimilation; backcrosses to a parental strain heterozygous for Hsp90 mutation restored the connection between these traits and Hsp90 function (141).

Hsp90-potentiated traits can also be genetically assimilated by repeated selection, but in this case assimilation occurs via acquisition of new mutations. What little is known comes from studies of Hsp90 potentiated resistance to azole antifungal drugs. Genetic assimilation of this trait was investigated in a series of *C. albicans* isolates taken over a two-year period from a patient receiving fluconazole therapy (36, 181). This series, and several others like it, represents a single strain evolving under selective pressure within a human host (181). Fluconazole resistance in early isolates was eradicated by impairing Hsp90 function pharmacologically or by growth at high temperatures. In striking contrast, later isolates possessed robust fluconazole resistance that was only modestly affected by changes in Hsp90 function or temperature. Fixation apparently occurred by the acquisition of new mutations that were unaffected by changes in Hsp90 activity. Thus, just as with Hsp90-buffered traits, repeated selection can lead to genetic assimilation of Hsp90-potentiated phenotypes. The precise molecular mechanism of these assimilations remains to be experimentally elucidated, but recent advances in whole-genome sequencing make this a tractable problem.

Traits that depend on *[PSI+]* can become genetically assimilated by further mutations or by reassortment of preexisting variation. For example, mutations in the stop codons that are suppressed in the prion state, or mutations affecting mRNA stability or promoter strength, could create the same phenotypes when *[PSI+]* is lost (165). However, mating experiments have shown that assimilation of *[PSI+]*-dependent traits can occur after a few generations, suggesting that reassortment of preexisting variation through recombination can have an important role. In general, however, molecular understanding of the mechanisms that govern genetic assimilation of buffered or potentiated traits is certainly needed.

HOW STRONG IS THE PROTEIN HOMEOSTASIS BUFFER?

Despite abundant evidence for the role of protein homeostasis in buffering, the nature and the extent of the buffer has remained elusive. However, recent studies suggest that the homeostasis buffer is surprisingly weak and its perturbation can be accomplished by expression of even a single aggregation-prone polypeptide. Gidalevitz et al. (64) examined the effect of expressing poly-Q, an aggregation-prone triplet repeat expansion protein associated with Huntington's disease (13). Whereas *C. elegans* expressing a temperature-sensitive muscle paramyosin mutant showed no defect

at the permissive temperature, coexpression of poly-Q resulted in failure to hatch or move in greater than 40% of organisms. Expression of poly-Q alone, or even a shorter nonaggregating poly-Q variant in combination with the paramyosin mutant, produced no such effect. Similar results were observed with other temperature-sensitive mutants (*perlecan*, *UNC-45*, and *ras*). In each case examined, expression of aggregation-prone poly-Q recapitulated the effects of growth at restrictive temperatures (64). This phenomenon was also observed by expression of another aggregation-prone protein, an amyotrophic lateral sclerosis (ALS)-associated mutant of superoxide dismutase (SOD1) (65). Thus, expression of even a single aggregation-prone protein can perturb cellular protein folding capacity sufficiently to prevent other unstable proteins from acquiring active conformations. In a broader context, the results offer insights into human disease progression and penetrance, and how proteostasis might mediate these processes. Polymorphisms that affect protein folding, which in humans amount to thousands in each individual (119), could collectively modulate the onset and symptoms of many diseases.

The requirement for protein homeostasis has also left an intriguing fingerprint in most genomes. In both yeast and bacteria, expression level is the most powerful predictor of a gene's evolutionary rate, with highly expressed proteins evolving more slowly (47, 127, 139). Drummond et al. suggested that the slow rate of change for highly expressed proteins arises from selection for translational robustness, i.e., sequences that fold correctly despite translational missense errors, preventing toxic protein aggregation (47, 48). Translational error rates are quite high ($\sim 5 \times 10^{-4}$ per amino acid in prokaryotes and eukaryotes) (51, 149), and therefore 20% of average-length (450 aa) proteins in eukaryotes are expected to have at least one misincorporated amino acid in their sequence (26, 47, 185). In the most extreme case of a three-megadalton protein (e.g., Titin), virtually every molecule should contain one or more translational missense errors. These errors increase the fraction of misfolded proteins and could amplify the destabilizing effects of new mutations, causing highly expressed proteins to evolve more slowly.

DOES NATURAL SELECTION FAVOR ROBUST LANDSCAPES?

The buffering and potentiation of genetic variation is pervasive and clearly has adaptive value in some circumstances. Has natural selection acted to produce any buffering or potentiating system per se? Or is it simply that many such mechanisms are beneficial for other reasons and contribute to buffering only as a secondary effect?

For example, in addition to buffering the effects of recessive mutations, diploidy has other advantages that have been extensively debated since the birth of the modern synthesis (28, 88, 123, 125, 131, 159, 192, 193). Diploid yeast cells, for example, adapt more quickly than haploids to novel environments in small populations because adaptation in this case is limited by the supply of raw material for evolution, i.e., new mutations (193). However, diploidy is not unconditionally favorable. The burden of diploidy is the increased mutation load; in equilibrium, it is higher in diploids than haploids. Given that most mutations are deleterious, the mean fitness of diploids is lower (124). Computer simulations do suggest, however, that diploidy could be advantageous because of its buffering function in certain circumstances, notably if the recombination rate is high and if deleterious alleles are generally recessive (123, 125, 131).

In contrast, the buffering effects of gene duplication are likely insufficient to explain their maintenance over evolutionary time. In very large populations, mutation rates must be identical at each duplicated locus or the copy subject to greater mutation load will eventually be silenced. In smaller populations, some fitness defect in a heterozygote is required for masking effects to drive maintenance of gene duplication (126, 136). Although buffering is unlikely to be an important factor in maintaining most

duplications (120), a potentially notable exception may found in the bacterium *Deinococcus radiodurans*. Its extreme resistance to genotoxic insult has been proposed to arise from multiple genome copies (37), although this hypothesis remains to be validated experimentally.

However, simulations suggest that in some cases buffering mechanisms that create mutational robustness could be selected for that purpose. Wilke et al. (184) evolved digital organisms with varying rates of mutation and showed that high mutation rates led to the "survival of the flattest," i.e., those genotypes that were located in relatively flat fitness peaks. Winning genotypes could tolerate mutations better but, as a trade-off, had a slower replication rate. The survival of the flattest has been experimentally confirmed in plant viroids and RNA viruses that have high mutation rates (32, 109). Although such experiments have not been performed in higher organisms with sexual life cycles, theoretical models suggest that mutational robustness can evolve as a result of sexual reproduction (7).

Whether biological network structures are a result of direct selection is similarly unclear. For example, genetic drift in organisms with relatively small effective population sizes could also explain the modularity of transcriptional enhancers without invoking natural selection (100, 101). This does not preclude the possibility that natural selection has shaped the modularity of transcriptional regulation, but it clearly illustrates the difficulty of separating adaptive and nonadaptive causes when the evidence has historically been primarily comparative and descriptive. However, computer simulations have shown that natural selection can promote modularity under many realistic scenarios (53, 85, 86). In contrast, the emergence of scale-free networks can be explained with an elegant model incorporating only two assumptions. First, networks grow continuously by adding new nodes. Second, new nodes are preferentially attached to existing nodes that already have many connections (9). Because this model applies to a wide variety of nonbiological scale-free networks that have not been subjected to evolutionary processes, it is very likely that the robust network structure is an emergent property of its growth pattern rather than an adaptation (9).

The very notion that natural selection could act to shape a phenotypic capacitor such as Hsp90 has been greeted with a significant amount of skepticism, mainly because higher-level selection is often invoked. Whether higher-level selection is an important evolutionary force remains a contentious issue in evolutionary biology (154, 186). Nonetheless, the question has remained whether Hsp90 has evolved specifically to act as a system for evolutionary capacitance. In our view, there is no reason to postulate that it should have. First, the protein-folding problem is as ancient as life itself. All known organisms have stress-regulated proteins specifically devoted to ensuring proper protein folding and function. Hsp90 is no exception. It is highly conserved—from bacteria to humans—and required for viability in all eukaryotes studied so far. It is critically needed for maturation and stability of a wide variety of proteins and is among the most connected proteins in the molecular interaction networks (182, 194). Unquestionably, this is the main cellular function of Hsp90. It is virtually impossible to separate the essential chaperone function of Hsp90 from its role in buffering, and thus the question of its evolutionary origin remains in the realm of speculation. Nonetheless, the question of whether Hsp90 can contribute to evolutionary change has already been answered: Hsp90 enables the rapid acquisition of antifungal drug resistance in diverse fungi (36). This scenario is highly relevant to both modern medicine and classical evolutionary biology. The remaining issue is the extent and type of Hsp90-dependent evolutionary change in nature.

The connections between environmental stresses, perturbations in protein homeostasis, and the phenotypic manifestation of genetic diversity provide robust systems for the inheritance of environmentally acquired characteristics. These mechanisms are plausibly based on known properties of biological systems and

operate within the Darwinian framework of mutation and natural selection. In this light, the wholesale dismissal of environmentally acquired traits by the framers of the modern synthesis of evolutionary theory seems unwarranted. Elucidation of detailed molecular mechanisms responsible for the appearance of specific traits and their subsequent assimilation, however, stands as a key objective for future studies.

SUMMARY LIST

1. Organisms can be remarkably resistant to phenotypic change. This is a result of many cellular mechanisms that ensure the stability of the phenotype against both environmental and genetic perturbations.
2. Most mutations are recessive, which means that diploidy and gene duplications provide backup copies of functional genes. Cellular pathways can also be highly redundant. Furthermore, the scale-free architecture of biological networks renders them tolerant to many perturbations.
3. Molecular chaperones have a central role in determining how genetic variation is translated into phenotypic diversity. On the one hand, they can buffer genetic variation, preventing it from having phenotypic consequence. On the other, they can allow the immediate phenotypic manifestation of certain new genotypes by facilitating the folding of novel variants or by preventing the deleterious secondary effects of mutations. Thus, chaperones can also potentiate the evolution of new traits.
4. In particular, the Hsp90 chaperone in eukaryotes and the GroEL chaperone in prokaryotes have been broadly implicated in buffering and potentiation of genetic variation.
5. Although robustness might seem to prevent adaptation to novel environments, it can actually facilitate adaptation by allowing populations to accumulate a large number of phenotypically neutral genotypes. These can place an individual closer to new fitness peaks, which can be reached by additional genetic or environmental change.
6. Environmental stress is a major contributor to genetic and phenotypic variation in two fundamental ways. First, it can increase the amount of genetic variation available for selection by increasing the rate of sexual reproduction, recombination, and mutation. Second, environmental stress can also affect the phenotypic expression of genetic variation because it is intimately linked to protein homeostasis.
7. In yeast, prions are emerging as an important bet-hedging mechanism for the inheritance of new phenotypes, based on stress-induced changes in cellular proteostasis, that lead to heritable, self-perpetuating changes in protein folding and function.

DISCLOSURE STATEMENT

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NOTE ADDED IN PROOF

Recent studies in *S. cerevisiae* provide robust evidence that Hsp90 exerts a strong influence on evolutionary processes. In strains from diverse ecotypes, Hsp90 determined the adaptive value of ~20% of standing genetic variation. It potentiated variants and buffered them with roughly equal

frequency. Polymorphisms responsible for diverse Hsp90-contingent traits occurred in proteins Hsp90 helps to fold, those it does not, and even in noncoding regulatory sequences.

Perhaps most tellingly, even modest reduction in Hsp90 function (elicited either pharmacologically or by a moderate environmental stress) improved the correlation between genotype and phenotype across more than 100,000 polymorphisms in 48 sequenced yeast strains. This establishes for the first time that Hsp90 has played an important role in shaping the evolution of current genomes (1).

1. Jarosz DF, Lindquist SL. 2011. Hsp90 and environmental stress transform the adaptive value of natural genetic variation. *Science*. In press

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Contents

Errata

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