Under cover: causes, effects and implications of Hsp90-mediated genetic capacitance

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Summary
The environmentally responsive molecular chaperone Hsp90 assists the maturation of many key regulatory proteins. An unexpected consequence of this essential biochemical function is that genetic variation can accumulate in genomes and can remain phenotypically silent until Hsp90 function is challenged. Notably, this variation can be revealed by modest environmental change, establishing an environmentally responsive exposure mechanism. The existence of diverse cryptic polymorphisms with a plausible exposure mechanism in evolutionarily distant lineages has implications for the pace and nature of evolutionary change. Chaperone-mediated storage and release of genetic variation is undoubtedly rooted in protein-folding phenomena. As we discuss, proper protein folding crucially affects the trajectory from genotype to phenotype. Indeed, the impact of protein quality-control mechanisms and other fundamental cellular processes on evolution has heretofore been overlooked. A true understanding of evolutionary processes will require an integration of current evolutionary paradigms with the many new insights accruing in protein science. *BioEssays* 26:348–362, 2004.
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"The constancy of the wild-type must be taken as evidence of the buffering of the genotype against minor variations not only in the environment in which the animals developed but also in its genetic make-up."

—C.H. Waddington[1]

Introduction

Evolutionary changes draw on phenotypic diversity among individuals in a population. Phenotypic diversity rests on the interaction of genetic and environmental factors. We now can discern effects of single genetic loci on phenotype, but effects of gene–gene and gene–environment interactions are far more complex. We have only begun to probe the molecular bases of multifaceted phenotypic variance. Since highly connected genetic networks can stabilize phenotypes,[2] one could hypothesize that disturbance of central network nodes will result in phenotypic variation. An environmentally responsive central node may provide a reversible trigger to disturb an otherwise robust network, producing varied phenotypes while the underlying genome remains unchanged. Molecular chaperones, which influence the conformation of many other proteins, are prime candidates for such environmentally responsive, highly connected molecules. Indeed, two recent studies[3,4] reported that the chaperone Hsp90 may have both large and small effects on many different phenotypes in flies and plants. These phenotypes may in part arise from stochastic effects. Notably, they can also be due to pre-existing genetic polymorphisms whose effects on pathway function are normally hidden. When Hsp90's buffer capacity is taxed, the function of normal pathways is perturbed; these hidden variants are revealed, and the phenotype is altered.

The fact that challenges to Hsp90 function can reveal hidden genetic variation now has unequivocal experimental support in evolutionarily distant lineages. Whether this finding has wide-ranging implications for novel trait evolution and/or phenotypic robustness remains controversial. Herein, we review the existing empirical evidence and hope to correct common misconceptions about chaperone-mediated buffering of genetic variation. Most importantly, we wish to engage biologists from all fields, with emphasis on future empirical studies.

Hsp90 as an essential eukaryotic chaperone

Molecular chaperones were originally defined as proteins that assist other polypeptides in folding and prevent unproductive interactions without becoming part of a final structure.[5] Chaperones are crucial in many cellular processes including folding of nascent polypeptide chains, protein translocation across membranes and protein degradation. The best-understood chaperones, Hsp70 and Hsp60, share the ability to recognize and transiently bind exposed hydrophobic residues that are characteristic of unfolded or incompletely folded proteins. They prevent improper protein interactions in the crowded cytoplasm. This function is of even greater

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importance under conditions that promote protein unfolding and aggregation, such as environmental stress.[6] Chaperones differ in their substrate specificity and recognition, the nature of their interactions and the type of product released. The boundaries of chaperone function are broadening and definitions are blurring,[7–10] but their essential role in guiding protein folding and maintaining function is evident.

Hsp90 is unusual among chaperones due to the diverse but select nature of its substrates (Hsp90 client proteins). Most are key regulators of growth and development. Hsp90 itself is one of the most abundant cytosolic proteins in eukaryotes, even in the absence of stress. In all tested eukaryotes, it is essential for viability and further induced during stressful conditions.[11–13]

The molecular weights and names of Hsp90 homologs differ between organisms but, for simplicity, we refer to all as Hsp90. The essential function of Hsp90 is conserved among eukarya. For example, when the mammalian glucocorticoid receptor is expressed in yeast or plants, which lack such a protein, it folds and functions properly through interaction with the endogenous Hsp90 proteins.[14]

The best-characterized substrates are steroid hormone receptors, kinases such as v-src, and transcription factors such as the heat-shock factor.[12] Hsp90 client proteins are often structurally flexible, allowing for transduction of signals via conformational changes induced by ligand binding, translocation, phosphorylation, or assembly. Since many different regulatory proteins depend on Hsp90, multiple pathways are sensitive to decreases in its activity.

The inherent conformational instability of Hsp90's client proteins is key to their interaction with the chaperone, as substrates lack a common sequence or structural motif. Indeed, substituting even a few amino acids can dramatically alter dependency on Hsp90; for example, the oncogenic kinase v-src is far more dependent on Hsp90 than its close homolog c-src.[15] Theoretically, any protein that acquires a metastable fold through stress or mutation may become dependent on Hsp90, as indicated by studies of related bacterial luciferases[16] and ErbB-1 mutants.[17]

Hsp90 and buffering of genetic variation

Recent publications reveal an unexpected consequence of Hsp90's central role regulating growth and development. When Hsp90's function is taxed, diverse pathways become sensitive to the effects of cryptic genetic variation. That is, Hsp90 acts as a buffer or capacitor of genetic variation, which accumulates under normal circumstances with little phenotypic consequence and is revealed in times of stress.

In the fly *Drosophila melanogaster*[3] and the plant *Arabidopsis thaliana*,[4] an extraordinary variety of phenotypes were observed when Hsp90 function was impaired by pharmacological inhibition or genetic manipulation, or its capacity was taxed by moderate environmental stress. Most individuals appeared normal, but some exhibited altered phenotypes. Different individuals had different defects, collectively affecting virtually every visible structure (Fig. 1). Many manipulations can produce pleiotropic phenotypes in different genetic backgrounds, but the number and variety of phenotypes revealed was remarkable. Most importantly, the particular phenotype depended on the individual's genetic background in both *Arabidopsis* and *Drosophila*.

Rutherford and Lindquist examined the genetic architecture of two Hsp90-dependent abnormalities (deformed eye and wing-vein phenotypes) with selection experiments. After several generations of selection for the traits, both were nearly fixed. Realized heritability values for replicate lines were non-zero, indicating that selection had acted on pre-existing genetic variation. Notably, the expression of both traits diverged between replicate lines. When individual chromosomes were introduced into a control genetic background, predisposition to the selected phenotype differed. Together, the divergence of phenotypes, differing heritabilities and dissimilar chromosomal contributions prove that expression of the trait depended upon multiple pre-existing polymorphisms.

To confirm the genetic basis of the deformed eye trait, lines selected for high penetrance were outcrossed to unselected strains. Penetrance in the progeny dropped to very low levels, establishing that the underlying factors are genetic rather than epigenetic. If inheritance had been epigenetic, an outcross would have resulted in maintenance of trait penetrance in the progeny.

Surprisingly, selection for an Hsp90-dependent trait could cause it to become Hsp90-independent. This finding is fundamental to the possible evolutionary consequences of the Hsp90 buffer. Rutherford and Lindquist selected solely for the altered trait, not for the mutant Hsp90 allele. After several generations of selection, none of the 70 flies genotyped harbored mutant Hsp90, though the selected trait was near fixation. Thus, temporarily compromising Hsp90 function was sufficient to reveal the effects of genetic polymorphisms. These could be enriched by selection until the trait was expressed even when Hsp90 function was restored—the trait had become robust and independent of fluctuations in Hsp90 function (Fig. 2).

The *Arabidopsis* study extended the concept of Hsp90 buffering beyond genetic variation in morphological traits. Exploiting unique advantages of the plant system, the authors quantified Hsp90 buffering in environmental response pathways and demonstrated a role for Hsp90 in plasticity and environmental canalization.

Arabidopsis thaliana allows exploration of Hsp90 buffering in ways not possible in other model organisms. First, *Arabidopsis*'s sessile lifestyle necessitates intricate links to environmental cues, facilitating study of the interplay between Hsp90 buffering and phenotypic plasticity (gene by environment interaction). Unlike flies, plants develop continuously throughout life, likely enhancing the importance of an en-

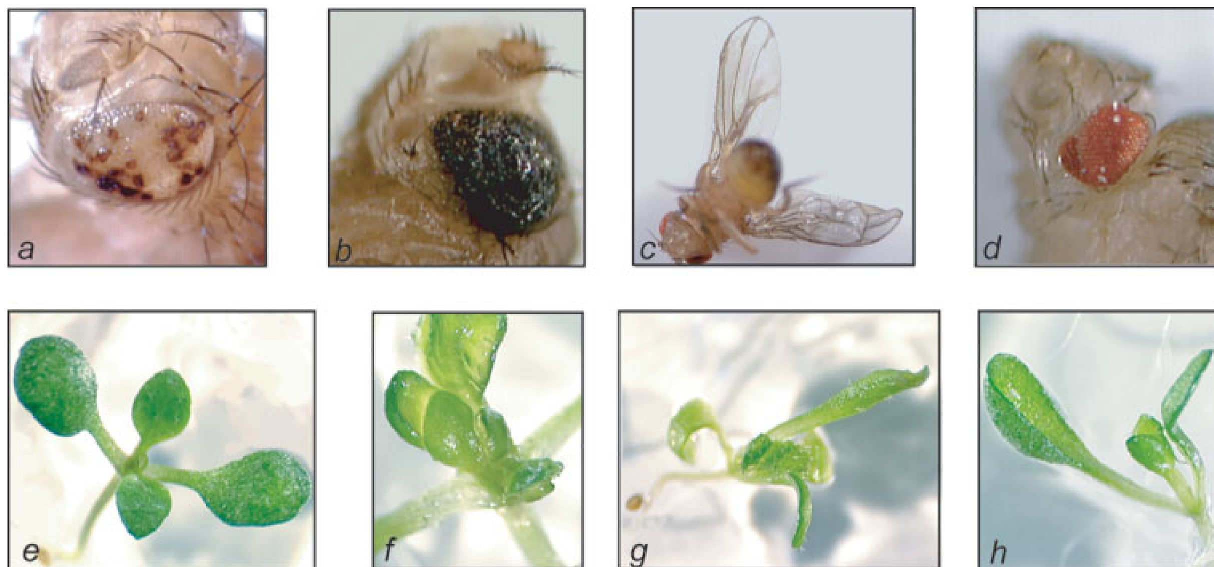


Figure 1. Diverse phenotypes associated with Hsp90 impairment in *D. melanogaster* (**a–d**) and *A. thaliana* (**e–h**). **a:** Smooth eye with black facets (impairment by heterozygous Hsp90 mutation). **b:** Black-facet phenotype increases in severity with increasing impairment of Hsp90 (Hsp90 mutations in heteroallelic combination). **c:** Thickened wing veins (heterozygous Hsp90 mutation). **d:** Deformed eye (Hsp90 inhibitor, geldanamycin). **e:** Untreated *A. thaliana*. **f–h:** Impairment of Hsp90 by geldanamycin affecting emergence of leaves (f), shape of leaves (g), and shape of cotyledons (h). All these phenotypes are dependent on the genetic background of the organism.

vironmentally responsive buffer. Second, due to the plant's inbreeding nature, near-homozygosity within *Arabidopsis* laboratory stocks is the norm. The effortless production of numerous clonal seeds without noticeable inbreeding depression allows the response of an identical genotype to be tested under many conditions. Third, genetically divergent wild lines have been collected. Crosses of these lines can easily produce hundreds of identical, but genetically heterozygous, F_1 progeny to explore genome epistasis and developmental homeostasis. Fourth, by self-propagating single seeds of segregating F_2 populations for eight generations, recombinant inbred lines (RIL) have been produced for complex trait mapping purposes.[18] Each RIL represents a different, but nearly homozygous, mosaic of the parental genomes. Thus, *Arabidopsis* provides a perfect system to disentangle genetic and environmental contributions to the phenotypes produced when Hsp90 buffering capacity is taxed.

To test if manipulation of Hsp90 uncovers genetic variation in plants, we used RILs derived from crosses of the Cape Verde Island and Landsberg *erecta* accessions. Should reduction of Hsp90 buffer capacity reveal pre-existing polymorphisms, one would expect Hsp90-buffered phenotypes to differ between lines but to be shared among most individuals of a given line. Indeed, our experiments consistently produced this result.[4]

To determine whether Hsp90 links environments and developmental response pathways, we assessed a classic example of plant phenotypic plasticity: hypocotyl elongation in dark grown seedlings. We quantified changes in hypocotyl elongation caused by pharmacological Hsp90 inhibition in numerous inbred lines.[4] In addition, we examined effects of Hsp90 inhibition on germination, root elongation and gravitropism in the dark, as well as greening after subsequent light exposure. Notably, different inbred lines were strongly affected in a particular plastic trait; furthermore, within a given line, different plastic traits were differentially affected. This result excludes drug toxicity as a cause of the observed effects and quantitatively establishes that revealed genetic factors influence one trait but not another. Thus, interference with Hsp90 buffer capacity can reveal variation in environmental response pathways as well as in morphological development pathways. Our other central findings relating to developmental homeostasis and environmental buffering[4] will be reviewed below.

Genetic variation is revealed by altered environmental conditions

For the storage of buffered variation to be significant in evolutionary processes, a natural mechanism allowing release must exist. The Hsp90 buffer has such a release mechanism: Hsp90 is an environmentally responsive protein. Consequently, Hsp90's buffer capacity can be altered by environmental change without any necessity for mutation of Hsp90 itself. Therefore, expression of cryptic polymorphisms is expected to

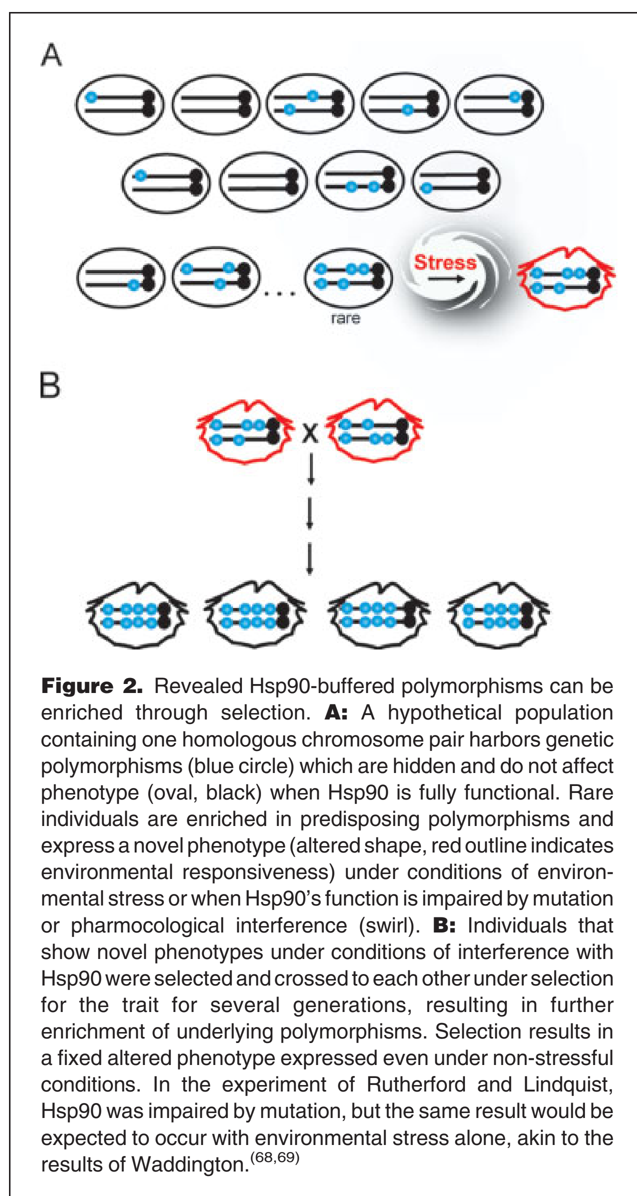


Figure 2. Revealed Hsp90-buffered polymorphisms can be enriched through selection. **A:** A hypothetical population containing one homologous chromosome pair harbors genetic polymorphisms (blue circle) which are hidden and do not affect phenotype (oval, black) when Hsp90 is fully functional. Rare individuals are enriched in predisposing polymorphisms and express a novel phenotype (altered shape, red outline indicates environmental responsiveness) under conditions of environmental stress or when Hsp90's function is impaired by mutation or pharmocological interference (swirl). **B:** Individuals that show novel phenotypes under conditions of interference with Hsp90 were selected and crossed to each other under selection for the trait for several generations, resulting in further enrichment of underlying polymorphisms. Selection results in a fixed altered phenotype expressed even under non-stressful conditions. In the experiment of Rutherford and Lindquist, Hsp90 was impaired by mutation, but the same result would be expected to occur with environmental stress alone, akin to the results of Waddington.[68,69]

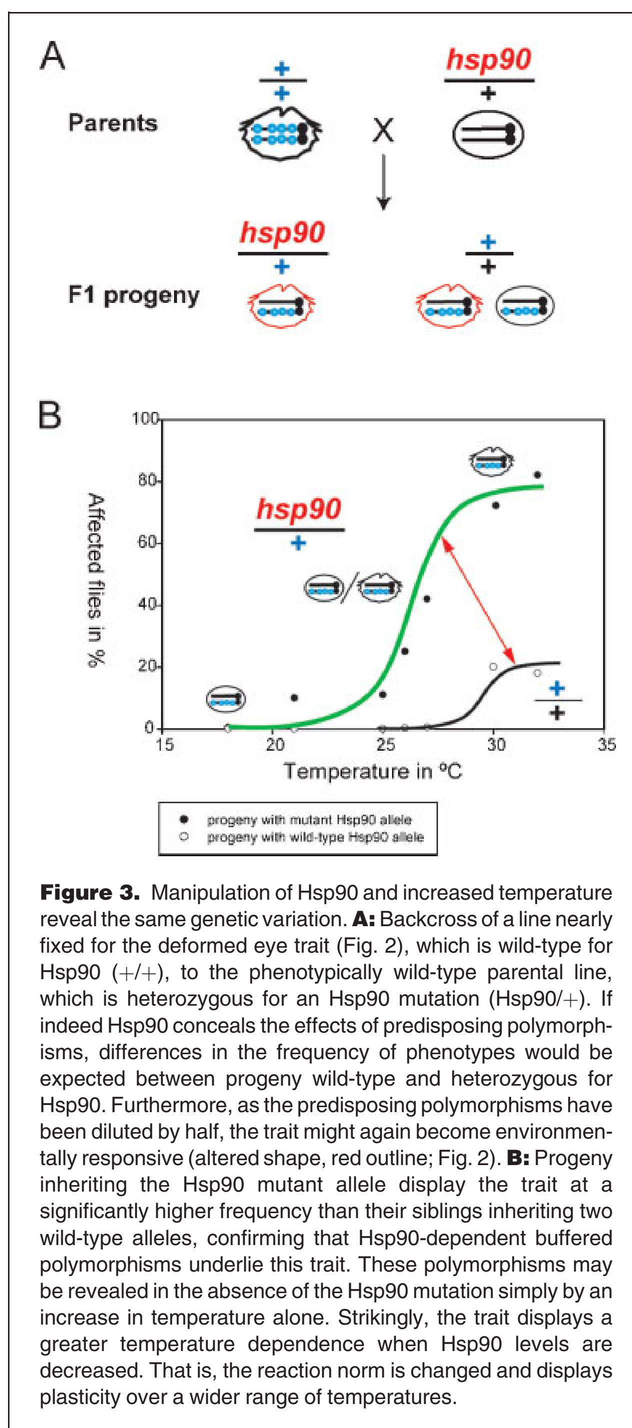


Figure 3. Manipulation of Hsp90 and increased temperature reveal the same genetic variation. **A:** Backcross of a line nearly fixed for the deformed eye trait (Fig. 2), which is wild-type for Hsp90 (+/+), to the phenotypically wild-type parental line, which is heterozygous for an Hsp90 mutation (Hsp90/+). If indeed Hsp90 conceals the effects of predisposing polymorphisms, differences in the frequency of phenotypes would be expected between progeny wild-type and heterozygous for Hsp90. Furthermore, as the predisposing polymorphisms have been diluted by half, the trait might again become environmentally responsive (altered shape, red outline; Fig. 2). **B:** Progeny inheriting the Hsp90 mutant allele display the trait at a significantly higher frequency than their siblings inheriting two wild-type alleles, confirming that Hsp90-dependent buffered polymorphisms underlie this trait. These polymorphisms may be revealed in the absence of the Hsp90 mutation simply by an increase in temperature alone. Strikingly, the trait displays a greater temperature dependence when Hsp90 levels are decreased. That is, the reaction norm is changed and displays plasticity over a wider range of temperatures.

respond to changes in temperature or other factors affecting protein folding. Indeed, in *Drosophila* and *Arabidopsis*, altered morphologies specific to Hsp90-dependent genetic variation were uncovered by temperature change.

Drosophila lines selected for high penetrance of the deformed eye trait and wild type for Hsp90 were backcrossed to the parental line containing a heterozygous Hsp90 mutation. All progeny would have about half the number of predisposing polymorphisms; 50% would be heterozygous for the Hsp90 mutation while the other 50% would carry both wild-type alleles. At normal temperature (Fig. 3), the progeny receiving two wild-type alleles rarely displayed the trait, whereas heterozygous progeny expressed the trait at a significantly higher frequency.[3] At moderately elevated temperatures, the trait penetrance in the offspring containing the mutant Hsp90 allele increased to a frequency observed before the outcross, suggesting that the combination of increased temperature and Hsp90 reduction resulted in functional dominance of the underlying heterozygous factors. Notably, at elevated tem-

peratures, a substantial number of flies carrying two wild-type Hsp90 alleles also expressed the same specific trait. Thus, environmental change alone can expose genetic variation also uncovered when Hsp90 function is selectively compromised by mutation.

The fact that Hsp90 need not be mutated to uncover genetic variation is central to the hypothesis that the Hsp90 buffer might play an important role in evolutionary processes. Due to Hsp90's many essential functions, constitutive impairment would reduce fitness and be unlikely to persist in wild populations. Even in the laboratory environment, Hsp90 mutations are rapidly lost when not deliberately selected for.[3] Rather, a reversible environmentally mediated alteration of Hsp90 functional capacity is sufficient to reveal the effects of accumulated variation. Such environmental fluctuations are far more likely to occur in nature than Hsp90 mutations. The reversibility of environmental challenges to Hsp90 function fundamentally distinguishes storage and release of Hsp90-buffered variation from other suggested buffering mechanisms. Intrinsic buffering by a genetic network, for example, requires additional irreversible mutations to reveal the stored polymorphisms.[19]

Hsp90 and buffering of epigenetic phenomena

Sollars et al.[20] expanded the scope of Hsp90 buffering to epigenetically determined traits. The authors used a *Drosophila melanogaster* stock harboring a dominant gain-of-function mutation of *Krüppel* (*Kr$^{If\text{-}1}$*), a zinc-finger transcription factor required for abdominal patterning. Mutant flies exhibit an irregular pattern of eye facets, presumably caused by ectopic expression of *Krüppel*. An enhancer screen identified ten different mutations that produce abnormal outgrowth of tissue adjacent to the eye and development of extra bristles. Notably, mutations at these loci affected outgrowth only when maternally inherited. Most were previously identified as members of the *trithorax* group, several of which form chromatin remodeling complexes.[21] Remarkably, five Hsp90 alleles had the same effect on ectopic outgrowth.

When highly inbred *Kr$^{If\text{-}1}$* flies were raised on food containing the Hsp90 inhibitor geldanamycin, ectopic outgrowth was observed. Strikingly, once the trait was established, selection increased its penetrance without further Hsp90 impairment. Similar results were obtained when a mutated *trithorax* group member was used to establish the phenotype. Therefore, increased penetrance of ectopic outgrowth was likely due to an epigenetic effect mediated by altered function in either Hsp90 or *trithorax* group genes. Note, however, that the origin of the outgrowth phenotype is not purely epigenetic as it requires the predisposing mutant *Krüppel* allele.

Hsp90 and chromatin-remodeling factors may act independently or in concert in transcriptional regulation. Freeman and Yamamoto[22] established that Hsp90 and its co-chaperone p23 are required for disassembly of receptor-mediated transcriptional regulatory complexes. Fletcher and colleagues[23] attributed the same phenomenon to the action of the SWI/SNF complex of chromatin-remodeling proteins, many of which are *trithorax* group members. Likely, chaperones and chromatin-remodeling complexes both mediate disassembly of receptor complexes. We speculate that these observations might well be related to the results of Sollars and colleagues. Supporting this connection, the frequency of ectopic outgrowth was increased not only by mutations in Hsp90 and SWI/SNF chromatin remodeling factors but also by transcriptional mediator and fibroblast growth factor receptor mutants. We hypothesize elsewhere about possible molecular mechanisms underlying the epigenetic inheritance of this Hsp90-buffered trait.[24]

Sollars and colleagues established chromatin structure as another mechanism underlying the genesis of buffered traits. The relative contribution of epigenetic versus genetic mechanisms in a variety of Hsp90-dependent traits, and, indeed, the capacity of the two to work in concert is of great interest. Most importantly, can phenotype be heritably altered by modifying chromatin via environmental modulation of Hsp90 activity? If so, do all cases require an underlying genetic polymorphism, such as that in *Krüppel*?

What can be buffered and how can it be released?

When considering the possible significance of the Hsp90 buffer in generating phenotypic diversity, the diverse nature of the polymorphisms that might be uncovered when Hsp90's buffering capacity is challenged is important. Consider a simple part of a hypothetical pathway with one Hsp90 client protein (Fig. 4A). First, a polymorphism could be present in the Hsp90-dependent client protein itself, making the entire pathway susceptible to fluctuations in Hsp90 function (Fig. 4B). Second, polymorphisms could occur in proteins that were previously independent of Hsp90 but, as a result of destabilizing mutations, now require interaction with Hsp90 for full function (Fig. 4C). Third, polymorphisms might be present in other proteins, or even in promoters or RNA regulatory regions, that do not interact with Hsp90, but instead function at other steps of Hsp90-dependent pathways and are therefore affected by its activity (Fig. 4D,E). In all cases, the polymorphism is revealed because the pathway is already sensitized and subsequent reduction of Hsp90 function perturbs output enough to alter phenotype. Lastly, Hsp90 reduction can produce new phenotypes by revealing the combinatorial effects of individual variants (Fig. 4C–F).

Hsp90 is very abundant and is further induced under heat stress. How can the functional capacity of such a protein be overwhelmed to reveal cryptic polymorphisms? Several non-mutually exclusive mechanisms could contribute. None are based on a mutation in Hsp90, but rather on temporary, reversible modulations of the chaperone's functional capacity.

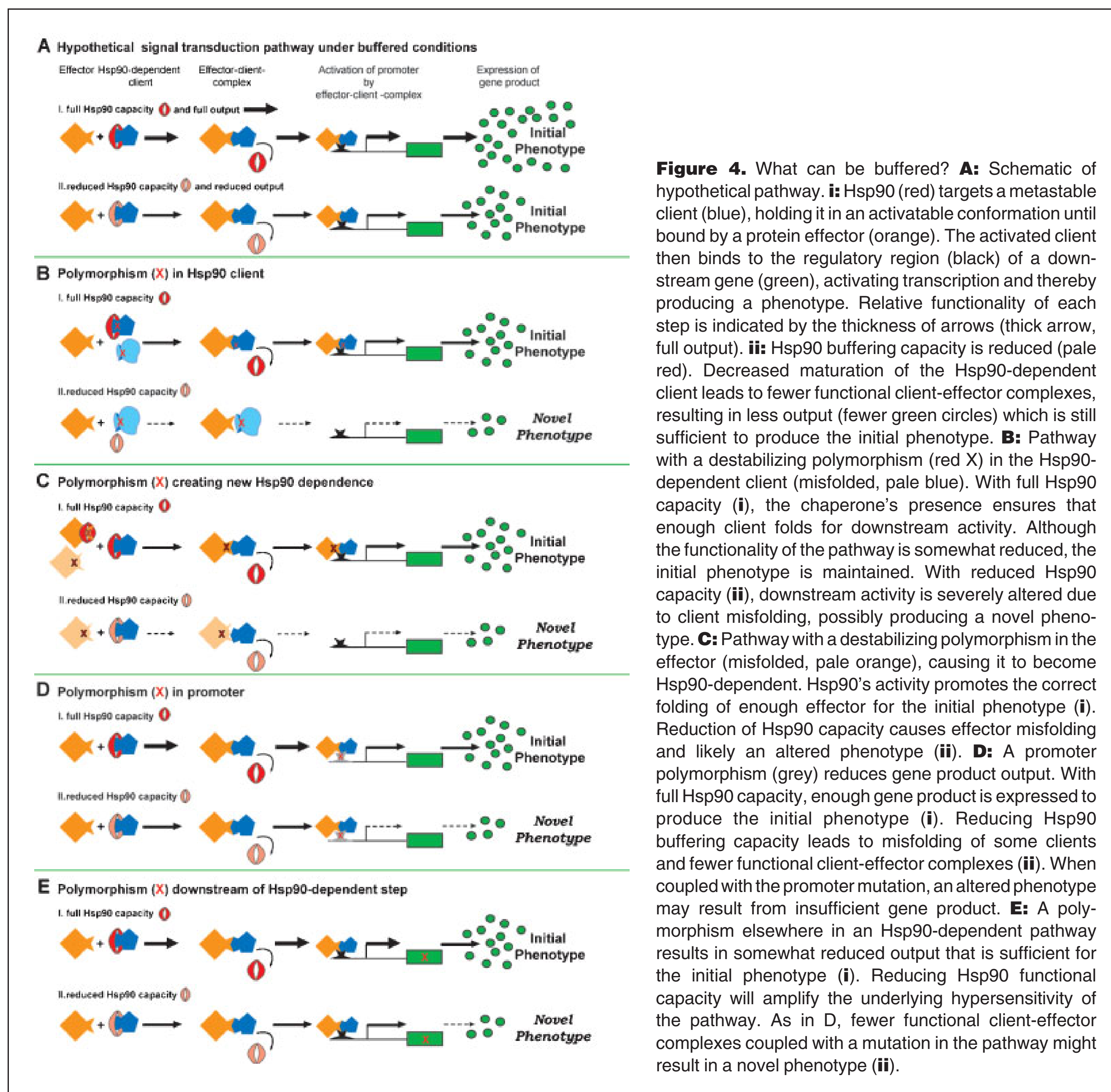


Figure 4. What can be buffered? **A:** Schematic of hypothetical pathway. **i:** Hsp90 (red) targets a metastable client (blue), holding it in an activatable conformation until bound by a protein effector (orange). The activated client then binds to the regulatory region (black) of a downstream gene (green), activating transcription and thereby producing a phenotype. Relative functionality of each step is indicated by the thickness of arrows (thick arrow, full output). **ii:** Hsp90 buffering capacity is reduced (pale red). Decreased maturation of the Hsp90-dependent client leads to fewer functional client-effector complexes, resulting in less output (fewer green circles) which is still sufficient to produce the initial phenotype. **B:** Pathway with a destabilizing polymorphism (red X) in the Hsp90-dependent client (misfolded, pale blue). With full Hsp90 capacity (**i**), the chaperone's presence ensures that enough client folds for downstream activity. Although the functionality of the pathway is somewhat reduced, the initial phenotype is maintained. With reduced Hsp90 capacity (**ii**), downstream activity is severely altered due to client misfolding, possibly producing a novel phenotype. **C:** Pathway with a destabilizing polymorphism in the effector (misfolded, pale orange), causing it to become Hsp90-dependent. Hsp90's activity promotes the correct folding of enough effector for the initial phenotype (**i**). Reduction of Hsp90 capacity causes effector misfolding and likely an altered phenotype (**ii**). **D:** A promoter polymorphism (grey) reduces gene product output. With full Hsp90 capacity, enough gene product is expressed to produce the initial phenotype (**i**). Reducing Hsp90 buffering capacity leads to misfolding of some clients and fewer functional client-effector complexes (**ii**). When coupled with the promoter mutation, an altered phenotype may result from insufficient gene product. **E:** A polymorphism elsewhere in an Hsp90-dependent pathway results in somewhat reduced output that is sufficient for the initial phenotype (**i**). Reducing Hsp90 functional capacity will amplify the underlying hypersensitivity of the pathway. As in D, fewer functional client-effector complexes coupled with a mutation in the pathway might result in a novel phenotype (**ii**).

The first, the "titration model", postulates that dramatic increases in the number of cellular targets of Hsp90 occur with stress, as more proteins exhibit the slightly destabilized state recognized by this chaperone. Hsp90 typically has a dynamic interaction with its targets, undergoing cycles of binding and release. Under stress, altered ATP:ADP ratios and the presence of new substrates with higher binding affinities may cause Hsp90 activity to become limiting for certain client proteins, even though Hsp90 is highly abundant. Indeed, this principle is thought to act as a gauge for cells to measure the folding state of proteins: the regulation of the heat-shock transcription factor (HSF) is effected by the titration of Hsp70 and Hsp90.[25–27]

Second, Hsp90-dependent, metastable proteins (either natural clients or new polymorphisms) are likely exquisitely sensitive to conditions that promote protein unfolding and limit Hsp90's ability to keep clients in an activatable state. This hypersensitivity may expose cryptic polymorphisms in connected pathways. Under these conditions, some destabilized proteins may irreversibly aggregate or be degraded, reducing

the function of entire pathways. Aggregation or phenomena such as narrow developmental windows may allow persistence of the altered phenotype in spite of stress-induced expression of Hsp90 or a return to ambient conditions.

Beyond cytoplasmic protein folding, titration and hypersensitivity may exert effects at all levels of organismic function. For example, taxing Hsp90 capacity may cause alterations in protein trafficking or degradation. Hsp90 has been implicated both in facilitating intracellular protein transport across mitochondrial membranes[28] and in partitioning proteins into folding or degradation pathways.[17,29–32] Limited Hsp90 may affect either system, causing proteins to be present in an inappropriate setting and increasing the chances for aggregation or improper interactions.

Conversely, such organismic functions may themselves induce titration or hypersensitivity. For example, impairment of degradation may overwhelm the chaperone machinery via accumulation of misfolded proteins; such a crisis will only worsen under stress. Studies of *Arabidopsis* plants[33,34] with reduced levels of the COP9 signalosome, implicated in the degradation of signaling proteins, provide circumstantial evidence for this hypothesis. These plants display a dramatic increase of polyubiquitinated protein, consistent with overloading the degradation system. This backlog may increase the number of cellular targets of Hsp90. Intriguingly, these plants exhibited diverse, low-penetrance phenotypes, strikingly similar to those observed with inhibited Hsp90.

Further, Hsp90 exerts little understood direct effects on gene expression. As discussed, deficiencies in Hsp90-mediated disassembly of regulatory complexes results in inappropriate persistence of transcription,[22] potentially enhancing effects of polymorphisms influencing transcriptional regulation. In addition, chromatin structure might be altered in a heritable manner, producing a novel epigenetically inherited trait.

All cellular protein-folding mechanisms are inextricably linked to each other. Thus, any perturbation in gene expression, RNA splicing, protein synthesis, protein trafficking, or protein degradation which can overwhelm the cellular protein-folding machinery and thereby influence Hsp90 buffer capacity may result in the uncovering of polymorphisms.

Is buffering specific to Hsp90?

If Hsp90's buffering capacity is inextricably linked to its chaperone function, then could any molecular chaperone buffer genetic variation? Certainly. Any chaperone could exhibit buffering activity, as chaperones generally protect other proteins from unfolding and subsequent aggregation. However, certain aspects of Hsp90's biology might render it particularly efficient as a genetic capacitor. First, unlike other chaperones, Hsp90 recognizes metastable folds rather than defined motifs of amino acids. As a contrasting example, Hsp70 recognizes hydrophobic stretches of four to five amino acid residues;[35–38] exposure of such core residues signals a nascent polypeptide chain or a globally unfolded protein domain. As such, Hsp70 is a generalist, with a multitude of substrates. Decreases in Hsp70 activity affect many substrates and generalized activities such as the folding of nascent polypeptide chains.[39] Consequently, the window between stresses that produce discrete outcomes specific to particular polymorphisms versus those that produce pleiotropic dysfunction may be narrow. Conversely, Hsp90 is not a main player in de novo protein folding[16] but instead chaperones a select set of client proteins. Therefore, limitations of Hsp90 availability specifically sensitize pathways with metastable elements. In *Arabidopsis*, challenges to Hsp90 function produce discrete phenotypic consequences before housekeeping activities are affected[4] (TS, CQ, SL unpublished observations).

Second, in some organisms, Hsp90 is present at higher levels than required for normal growth. In yeast, Hsp90 levels can be decreased by ten-fold with no effect on growth at normal temperatures.[40] In contrast, reduction of Hsp70 levels causes upregulation of heat-inducible chaperones, including Hsp70 itself.[41] The malleability of Hsp90 activity is an attractive feature for a genetic capacitor.

However, Hsp90 frequently functions in concert with Hsp70. Environmentally induced phenocopies of well-known developmental mutant phenotypes in *Drosophila* can be suppressed by Hsp70 overexpression,[42] suggesting buffering activity by this chaperone. Thus, we speculate that other chaperones contribute to buffering, but Hsp90's malleability and specificity likely render its buffering effects more dramatic in eukaryotic cells.

Interestingly, deletion of the Hsp90 homolog in *E. coli* is not lethal but results in a subtle thermosensitive phenotype.[43] In contrast, the chaperonin GroEL–GroES (GroE) complex is essential, presumably because it facilitates the folding of essential cellular proteins. GroEL is not required for folding most eubacterial proteins,[44] but many cytosolic proteins can become GroEL substrates if their folding is delayed. GroEL overexpression has been shown to buffer the fitness effects of deleterious polymorphisms in *E. coli*,[45] and GroE overexpression suppresses many mutations.[46,47] Thus, if buffer capacity is a byproduct of the biochemical function of the chaperone, whichever chaperone recognizes substrates destabilized by subtle polymorphisms might act as a buffer. Although buffering has not been investigated in Archaea and the relative importance of various folding mechanisms is unknown, it is tempting to speculate that a specialized chaperone with diverse substrates, properties similar to those of eukaryotic Hsp90 and prokaryotic GroEL, will show buffering abilities.

More Hsp90—better buffering?

If polymorphisms can be revealed by reduced availability of Hsp90, would increased levels confer more efficient buffering?

This question has yet to be addressed experimentally, but circumstantial evidence points to possible answers.

Since Hsp90's interaction with its substrates is dynamic, based on relative binding affinities, its levels may already be optimized. Indeed, overexpression of Hsp90 or other heat-stress proteins has been shown to slow development.[48–51] Increased availability of Hsp90 may shift the equilibrium of its interactions towards the client-bound state[13,52] and delay client protein maturation or transport. Given previous results on overexpression of other chaperones,[49,53] it may be difficult to substantially raise the levels of the already abundant Hsp90.

As an example of an alternative approach, one might correlate naturally occurring differences in Hsp90 expression to different lifestyles and buffer capacities. Such correlations might offer a starting point for further investigations. For instance, inbreeding and asexual species lack the general buffer provided by heterozygosity and suffer from increased fixation of slightly deleterious alleles.[54] Here, Hsp90 might play a more important role in genetic buffering. Increased buffer capacity in inbreeding species might diminish inbreeding depression on short evolutionary timescales, as suggested by the overexpression of GroEL observed in endosymbiotic but not free-living bacteria.[45] As increases in buffer capacity have associated costs, reductions of inbreeding depression cannot be maintained indefinitely by continual capacity expansion. Notably, inbreeding species have independently arisen in many lineages but rarely persist for long evolutionary periods.

A. thaliana, which switched to an inbreeding lifestyle recently in its evolutionary history, has four cytosolic Hsp90 copies, and two of these are products of recent duplication events (less than 6% nucleotide divergence).[55,56] Such duplications might allow functional diversification of the Hsp90s, eliminating possible biochemical drawbacks to overexpression.

Can buffered variation contribute to evolutionary change?

Natural selection acts on phenotypes, rather than genotypes. However, selection can only fix traits with an underlying genetic basis. With Hsp90 fully functional, the phenotypic consequences of genetic polymorphisms in many pathways are negated, rendering them neutral to selection. When buffer capacity is reduced, phenotypes arise from the previously concealed genetic variation, allowing selection on the underlying genetic variants.

This phenomenon has the potential to influence constraints on the pace and character of evolution. Note that the evolutionary consequences of the Hsp90 buffer derive solely from hidden genetic variation that can be exposed by environmental stress. We need not imply that Hsp90 buffering function has adaptive value itself. Rather, for this discourse, we assume that buffering is a byproduct of the chaperone's biochemical function. The evolution of systems solely to promote future "evolvability"—the ability to adapt more rapidly to novel circumstances—is viewed as theoretically unlikely[57,58] (for an alternative viewpoint, see refs. 59 and 60) since it would produce no immediately selectable phenotype.

The Hsp90 buffering system, however, possesses a selectable, essential biochemical function—to chaperone metastable proteins.[5] Since genetic buffering may arise as an inseparable consequence of Hsp90's biochemical activity, this particular aspect need not have been adaptively selected and may represent an evolutionary "spandrel".[61] The evolutionary effects of the Hsp90 buffer will depend on several currently unknown parameters, such as the rate at which buffered variation accumulates (a function of the underlying mutation rate and the percentage of total variation that can be buffered), the time between buffer release events, and the amount of the standing variation exposed each time the buffer is lowered. Clearly, all these parameters will vary for different organisms and environments.

The most basic question is the identity and nature of potentially buffered polymorphisms. Multiple experimental efforts are underway to address this issue; general conclusions must await the characterization of a large variety of buffered polymorphisms.

Other essential parameters are the frequency at which stored genetic variation is exposed to selection and the percentage of this variation released at each exposure. These factors will be affected by the magnitude of the stress required to cause release. We showed that different pathways have differing dependencies on Hsp90, as do different alleles within the same pathway.[4] Therefore, a very mild stress can reveal hidden variation in pathways that are highly dependent on Hsp90, while a much more severe stress may be required to uncover variation in less-dependent pathways. Combined, these factors relate to the central issue: how much hidden variation is accessible to selection?

At one extreme, very common stresses could cause constant uncovering of Hsp90 buffered variation. Hidden variation would not then accumulate in the genome to appreciable levels. At the other extreme, buffered variation would never be naturally released. Polymorphisms would then never become selectable. In either case, the Hsp90 buffer would be of little evolutionary consequence. Either extreme may apply in some organisms, but, in the two tested, several lines of evidence suggest that buffered variation can be naturally accumulated and released. First, recent wild isolates of both *Drosophila* and *Arabidopsis* show accumulation of buffered variation.[3,4] Many of the revealed phenotypes would undoubtedly be deleterious in the wild. Maintenance of these variants would be unlikely if constantly revealed. Second, the release conditions are not extreme—modest temperature increases (five degrees in *Arabidopsis*) can be sufficient.[3,4]

In contrast, release of stored variation at intermediate intervals should have greater evolutionary consequences.

First, consider release of buffered variation that occurs at fairly frequent intervals. In this case, accumulation of many buffered polymorphisms in a particular pathway is unlikely, so uncovering of single variants will predominate. Our preliminary results indicate that several fully recessive *Arabidopsis* mutant alleles become nearly completely dominant when the Hsp90 buffer is reduced.[62] A similar phenomenon has been observed in *Drosophila*, in which the uncovering of a recessive heterozygous mutation by high temperatures is dependent on heat-shock protein levels.[63]

Should any advantageous hidden variant acquire dominance via buffer reduction, its frequency is likely to increase rapidly as selection is far more effective on dominant alleles. This effect of the Hsp90 buffer would have the greatest impact when the desirable allele is at low frequency and most susceptible to loss from the population by genetic drift. Because of drift, even advantageous recessive alleles may need to arise independently several times before becoming established. Each mutation is a rare event; thus, an increase in penetrance of recessive alleles may dramatically hasten their fixation and speed phenotypic evolution.

Equally important, release of stored variation at intermediate frequencies may rapidly and efficiently purge the population of normally recessive deleterious alleles. Normally, this process is extremely slow for low-frequency recessive alleles as they rarely exert any phenotypic effect. Such punctuated selection will prevent affected alleles from achieving a steady-state equilibrium of mutation-selection balance.

Second, consider the release of buffered variation at fairly long intervals where multiple polymorphisms in a particular pathway may have accumulated and can be revealed. Such release may yield phenotypic diversity in highly robust, otherwise monomorphic traits. Such phenotypic alteration is initially unstable, as the population would revert to monomorphism if conditions return to normal. As demonstrated,[3] selection on novel variants may rapidly cause the traits to become Hsp90-independent, preventing reversion to monomorphism.

The synchronized exposure of multiple polymorphisms has the potential to allow substantial shifts in phenotype without passage through intermediate forms. Although most combinations will be deleterious, very rare ones may be advantageous. Such a model could suggest one solution to a classical evolutionary dilemma: how are phenotypic transitions that require multiple mutational steps achieved, when intermediate states have reduced fitness? In other words, since selection can only increase a population's fitness, how can a population that occupies a local maximal fitness peak on an adaptive landscape cross a valley of reduced fitness to reach the globally optimal highest fitness peak—how does adaptive evolution continue once a population reaches a local fitness optimum?

Without considering a buffer such as Hsp90, such peak shifts may occur through genetic drift, which can fix slightly deleterious alleles in small populations. In large populations, however, genetic drift is negligible. Furthermore, the simultaneous occurrence of the multiple mutations required to cross the adaptive valley is highly unlikely. Wright's classic solution is the shifting balance theory[64]—in a subdivided population of large size, certain subpopulations may be small enough for drift to effect the phenotypic transition. Once present in the subpopulation, the new advantageous phenotype may then invade the general population. Another mechanism posits the neutral accumulation of variation in a non-functionalized copy of a duplicated gene.[65] This variation is then transmitted to the functional copy by gene conversion, simultaneously exposing multiple polymorphisms. Recent simulations indicate rapid shifts to alternative adaptive optima are feasible through the uncovering of genetic variation buffered via networks.[19]

Unlike these mechanisms,[19,65] release of Hsp90-buffered variation is a genome-wide phenomenon linked to stressful environmental conditions. The buffered variation is released precisely under those challenging conditions when selection is most stringent and novelty might be most beneficial. As traits can become Hsp90-independent after only a few generations of selection, very short-lived extreme environmental changes or fluctuating environments may have an oversized evolutionary impact due to the storage and release of cryptic genetic variation.

Hsp90, plasticity and genetic assimilation

Phenotypic plasticity is the ability of a genotype to produce different phenotypes depending on environmental conditions. Plasticity of a particular genotype can be visualized in a reaction norm—the set of phenotypes produced by an isogenic genotype under diverse environmental conditions. Such reaction norms may be either non-plastic, with a single phenotype regardless of environment, or plastic, with multiple phenotypes depending on conditions. The reaction norm is also a temporal concept—organisms may be competent to respond to an environmental cue only at a given point in development. The amount, pattern, rapidity and reversibility of the response can all theoretically be altered by selection, as all are genetically determined.[66,67]

Hsp90 function can affect reaction norms in both *Arabidopsis* and *Drosophila*.[3,4] Rutherford and Lindquist used a range of temperatures to examine expression of the deformed eye phenotype in a population of siblings differing in the presence of an Hsp90 mutation. The reduction in Hsp90 function dramatically changed the reaction norm (Fig. 3). In effect, the mean plasticity of the genotype increased; it became more environmentally responsive.

These results hark back to classical experiments by Waddington. He observed that a crossveinless phenocopy arose in a wild-fly strain exposed to heat shock.[68] Through selection, this initially environmentally dependent trait lost

plasticity and was fixed. These and similar data suggested the inheritance of acquired characters. Waddington reconciled his results with Darwinian theory through his hypothesis of genetic assimilation.[68,69] According to Waddington, selection may enrich environmentally responsive polymorphisms predisposing an organism to a trait so that the trait loses plasticity and becomes assimilated (fixed).

The assimilation of Waddington's crossveinless phenotype is reminiscent of the assimilation of phenotypes Rutherford and Lindquist studied. Here, manipulation of Hsp90 not only generated plasticity from an established reaction norm, but also led to near fixation of an altered trait. Thus, manipulation of Hsp90 may allow the genesis of plasticity in a non-plastic trait and subsequent assimilation from a plastic state.

Could Hsp90's influence on plasticity and assimilation be more general? Previous studies have shown that heritability of many traits varies along reaction norms,[70–74] and, in particular, may increase under stressful environments.[75–78] For example, larval development in *D. melanogaster* is normally monotonically faster with increasing temperature. Selection for more rapid development failed at most temperatures. However, at heat-stress conditions (32°C), selection yielded heritable increases in development rate,[79] which persisted at both high and normal temperatures.

Reducing Hsp90 function also increases the heritability of plastic traits in *Arabidopsis*.[62] Plasticity in the dark response of hypocotyls was almost abolished in some genetic backgrounds but was little affected in others,[4] implying that the effects of Hsp90 function on plasticity depend on underlying polymorphisms. Notably, heritability increased significantly when Hsp90 was inhibited. Here, Hsp90 dependency is unlikely to be a snapshot of assimilation, as hypocotyl length plasticity is one of the few examples conclusively shown to be adaptive.[80]

Indeed, one organism has utilized Hsp90 as a central regulator to stabilize adaptive plasticity.[81] The parasite *Leishmania donovani* has two life stages: the promastigote stage in sandflies, and the amastigote in mammals. The temperature increase that occurs during transmission to the mammalian host triggers differentiation from the promastigote to the pathogenic amastigote. Pharmacological inhibition of *L. donovani* Hsp90 is sufficient to trigger this metamorphosis, suggesting that this parasite senses successful transmission through changes in Hsp90 buffering capacity. How *L. donovani* acquired Hsp90 dependence of its plastic response and whether such dependence evolved multiple times will yield insight into the partitioning of Hsp90's role in plasticity, buffering of genetic variation and signal transduction.

Hsp90 and developmental homeostasis

How is a phenotype stably expressed despite genetic and environmental variation? At the population level, canalization is a genome's ability to produce a regular phenotype regardless of genetic or environmental perturbation.[1] At the individual level, developmental stability is the ability of an individual to produce invariant repeated characters throughout development.[82] Many hypotheses on the origin of canalized states have been proposed and recently reviewed.[83–86] Here, we will focus on the implications of chaperone-mediated buffering for canalization.

Due to difficulties in defining a measure of canalization, few empirical studies exist. Thus, many decades after Waddington and Schmalhausen[87] independently proposed the concept, the question remains whether canalized states represent evolved or intrinsic properties of biological systems.

Several authors support an evolved origin of canalization, but debate whether canalization primarily results from redundancy of duplicated genes[86,88] or environmentally imposed stabilizing selection leading to the formation of complex regulatory networks.[89–92] In contrast, Siegal and Bergman[2] proposed that canalization is a fundamental, intrinsic feature of functional genetic networks. Both empirical and computational data agree that evolved networks are extremely robust to perturbation, and a more densely connected network is often associated with increased canalization.

The storage of hidden genetic variation by Hsp90 defines one molecular mechanism achieving genetic canalization of many traits simultaneously. However, whether Hsp90 influences environmental canalization is debated. According to Milton et al., Hsp90 function is unrelated to environmental canalization.[93] In several traits in *Drosophila*, Hsp90 manipulation did not affect fluctuating asymmetry (FA), which is a quantification of random, small deviations from bilateral symmetry within an individual. FA represents an intraindividual measure of developmental stability whose relationship to population-level environmental canalization is unclear.[94] In striking contrast, Hsp90 manipulation increases the variability of hypocotyl and root length in isogenic *Arabidopsis*.[62] Further, Hsp90 inhibition resulted in increased morphological variation within isogenic *Arabidopsis* accessions. Importantly, developmental homeostasis but not viability were greatly decreased when Hsp90 was modulated in isogenic F_1 progeny created by crossing divergent *Arabidopsis* accessions.[4] Extrapolating this result, stabilization via Hsp90 may aid the integration of related genomes, contributing to the viability and fitness of intraspecific and interspecific hybrids. In summary, fully functional Hsp90 acts as both a genetic and environmental canalizer in *Arabidopsis*, dampening the effects of stochastic events intrinsic to development[4] (Fig. 5). Such a multifaceted role has been proposed for canalizing mechanisms.[84]

The evolutionary consequences of the Hsp90 buffer would be negated if destabilizing consequences of removing environmental canalization outweighed advantageous effects of revealed genetic polymorphisms.[84] However, the phenotypic

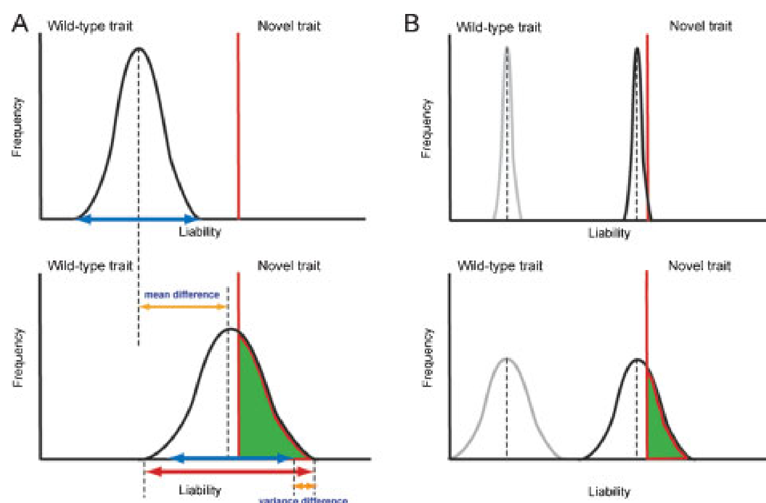


Figure 5. Population predisposition to an Hsp90-dependent threshold trait. **A:** Population with genetic variation. The underlying liability to the trait is affected both by genetic and environmental factors. With normal Hsp90 buffer capacity, the trait is not expressed (**top**). When Hsp90 buffer capacity is reduced, the liability shifts as cryptic predisposing factors are revealed (**bottom**). Individuals to the right of the threshold, represented by the red bar, express the novel trait (green shaded area). Both the mean and the variance of the liability are affected (orange arrows), but empirical evidence demonstrates that the difference in mean values may be an order of magnitude larger than the increase in variance. **B:** Inbred population without genetic variation. Differences in liability are solely due to environmental effects. Some populations fixed for different genetic variants will be more predisposed (black) to a trait than others (grey). When Hsp90 buffer capacity is normal (top), empirical evidence shows that rare individuals in predisposed populations may stochastically cross the threshold and display the trait. Reduction of Hsp90 buffer capacity amplifies the effects of stochastic decisions inherent in development and increases liability variance (bottom). Predisposed populations will display a novel trait at high frequency (black curve, green area), while others which are not predisposed will not display the trait even with reduced buffer capacity (grey population).

effect of Hsp90-dependent polymorphisms affecting hypocotyl length is an order of magnitude larger than the effects of increased variance[62] (TS, CQ, and SL, unpublished observations) (Fig. 5). Should such revealed polymorphisms be adaptive, their selective advantage would prevail over the decreased environmental canalization.

Interestingly, initial developmental instability has been experimentally linked to canalized trait evolution. For example, selection for insecticide resistance in the Australian sheep blowfly caused resistant flies to initially display increased developmental instability and reduced fitness in the insecticide's absence. Subsequently, a modifier in another gene arose that suppressed instability and restored fitness.[95] Could increased instability be an avenue to change the output (phenotype) of robust networks to produce novel phenotypes?

Because of Hsp90's environmental malleability and genetic buffering ability, the highly connected chaperone is a possible catalyst for both mediating initial instability and establishing novel network connections. Interference with Hsp90's capacity may reduce connectivity in complex networks, resulting in general destabilization. As uncovering cryptic polymorphisms occurs simultaneously with destabilization, new network connections may be formed. The altered pathway might produce a selectable phenotype, and the newly formed connections may restore robustness (Fig. 6).

Dependence on Hsp90 might add robustness to nascent networks with low inherent connectivity. Addition of further connections may reduce the network's dependence on Hsp90 for robustness. Indeed, recent simulations support the plausibility of this argument.[19]

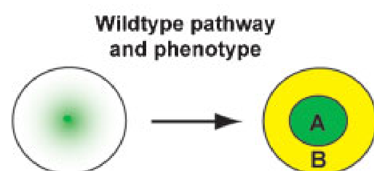
Hsp90 and cancer

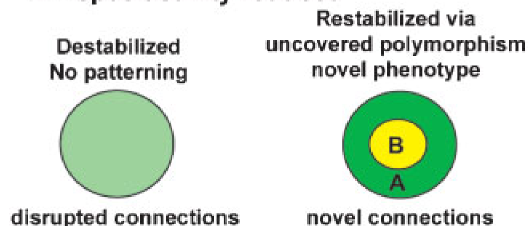
Many human solid tumors display extensive phenotypic heterogeneity and undergo dramatic clonal evolution during the progress of malignant progression. Most cancers arise via complex interactions between genetic and epigenetic mutations, environmental perturbations and stochastic processes; all are influenced by Hsp90 in model organisms.[96,97] Hence, Hsp90 might contribute to both tumor progression and oncogenic transformation, possibly in different ways. This protein is overexpressed in many cancers and the distribution between its free and complex-bound forms dramatically differs from normal cells.[52] Hsp90 chaperones various oncogenic kinases.[96,97] Consequently, cancer cells are exquisitely sensitive to interference with Hsp90.[98] This sensitivity might reflect an increased need for Hsp90-mediated client protein

A. Hypothetical embryonic patterning

I. Hsp90 fully functional

II. Hsp90 activity reduced

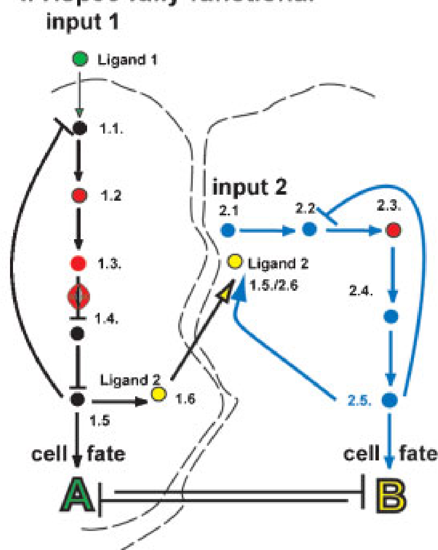




B. Novel connections via Hsp90-buffered polymorphisms

I. Hsp90 fully functional

II. Hsp90 activity reduced



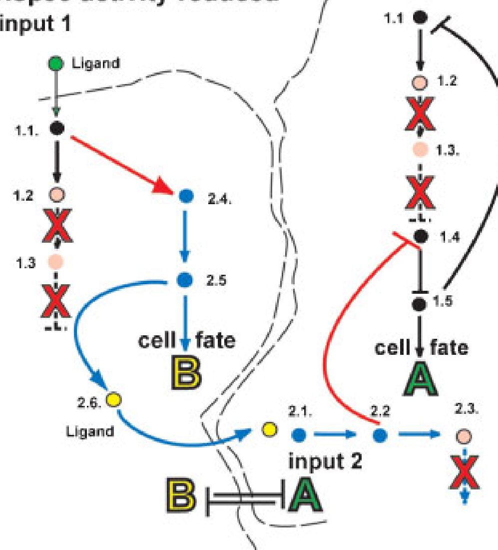


Figure 6. Hypothetical rare output switching of a robust network mediated by environmental destabilization and restabilization by uncovering of Hsp90-dependent polymorphisms. **A:** Phenotypic outcomes. **i:** Wild-type; an initial developmental gradient (green) leads to two cell fates, A (green) and B (yellow). **ii:** Environmental stress causes destabilization of the network and lack of patterning. Uncovering of Hsp90-dependent polymorphisms may restabilize the network and create a novel phenotype. **B:** Hypothetical network leading to the outcome depicted in A. **i:** Wild-type network. The initial gradient of the morphogen (1.1, green) is sensed at a threshold level by a genetic pathway leading to cell fate A. This pathway both inhibits itself and produces a second ligand (1.6, yellow), which travels extracellularly (dotted lines, cell membranes) and initiates a second pathway (blue arrows), leading to cell-fate B and further spread of the B-producing signal. Differentiation to fate A or B is irreversible; no cell can express both fates. Node 1.3 (red) is always Hsp90-dependent; nodes 1.2 and 2.3 (red with black outline) carry Hsp90-dependent polymorphisms segregating in the population. Destabilization of the network by loss of node 1.3 leads to no patterning. **ii:** Novel connections due to Hsp90-dependence may restabilize the network and lead to a different output. Upon a reduction in Hsp90 buffering capacity, nodes 1.2, 1.3 and 2.3 become non-functional (pink, dashed arrows with red X). Accumulation of high levels of active node 1.1 protein leads to a novel interaction with node 2.4 (red arrow). Similarly, accumulation of active node 2.2 protein causes a novel interaction with node 1.4 and an immediate stable reversal of the initial pattern. If favorable, the new pattern may become fixed in the population through selection on the Hsp90-dependent polymorphisms.

maturation in growing tumor cells, an increasingly hostile environment characterized by hypoxia or nutrient deprivation, a requirement for increased buffer capacity due to the mutations and genomic instability typical for tumor cells, or a combination of all of these.

Oncogenic transformation often involves dominant gain-of-function mutations.[99–101] Some oncogenic mutations cause increased instability and heightened dependence on Hsp90, for example, the oncogenic v-src in comparison to its regulated predecessor, c-src. Such mutants will lose function when Hsp90 becomes limiting. Indeed, low concentrations of geldanamycin can reverse some cancerous transformations.[102,103]

While Hsp90 may be involved in evolution of cancer lineages, mammalian homeothermy and the peculiarities of

mammalian development will limit the uncovering of genetic polymorphisms influencing organismic phenotype. Heat stress will rarely affect the developing embryo, but it is susceptible to other protein-folding stresses, such as ethanol, heavy metals or severe illness of the mother. In the absence of frequent release, buffered polymorphisms might accumulate, keeping buffer capacity close to its limit. Thus, interference with Hsp90 in mammalian embryos might produce pleiotropic developmental phenotypes instead of revealing phenotypes specific to particular buffered genetic variants.

It is unknown how other functional aspects of Hsp90, such as maintenance of developmental homeostasis, affect mammalian development. Hsp90-influenced disruptions of developmental homeostasis affecting morphology will predominantly occur in utero. In this context, it is noteworthy that reduced developmental stability, as measured by various sporadic congenital abnormalities affecting fluctuating asymmetry, is associated with early childhood cancer.[104] In summary, we speculate that Hsp90's influence on cancer transformation and tumor maintenance may mirror its demonstrated role in genetic and environmental canalization.

Conclusions

Phenotypic buffering by Hsp90 sheds light on the complex molecular processes between the inputs of genotype and environment and the output of phenotype. Our prior research has conclusively demonstrated that genetic variation can be stored and released as a consequence of Hsp90 function. In *Arabidopsis*, Hsp90 also stabilizes development against the effects of stochastic processes intrinsic to development. Thus, Hsp90 represents a molecular foothold to empirically address long-standing questions regarding the genesis of complex phenotypes.

Many questions await exploration: for example, what is the relative contribution of Hsp90 and other chaperones to genetic versus environmental buffering? Does such partitioning differ between groups of organisms—prokarya versus eukarya, unicellular versus multicellular, or poikilothermic versus homeothermic? Does Hsp90 stabilize certain lineages against environmental perturbation, thus becoming an environmental sensor and plasticity switch? Is Hsp90 an evolutionary capacitor in lineages where effects of revealed genetic variation outweigh effects on developmental stability?

Beyond a single environmentally responsive molecule, what other mechanisms might influence the expressivity and penetrance of pre-existing polymorphisms? The concept of Hsp90-mediated buffering might extend to any mechanism causing increased demand for the functionality of cellular building blocks, thus reducing the margin of error and potentially revealing cryptic variation by amplifying its effects. An obvious candidate for amplifying or negating effects of underlying polymorphisms is metabolic rate. For example, lower temperature, commonly associated with reduced metabolic rate, will diminish the effects of random mutagenesis in *E. coli*.[105] Biochemists routinely exploit low temperature to express mutant proteins, avoiding their misfolding and aggregation.[106]

The phenotypic effects of protein folding relate to the etiology of several devastating human diseases. Creutzfeld-Jakob, Huntington's, Alzheimer's, and Parkinson's diseases, to name a few, share the aggregation of a disease-specific protein. The most-common cystic fibrosis allele, ΔF508, encodes a protein with a temperature-sensitive folding defect.[107] Consequently, maintenance of proper protein folding and balance of the various chaperone activities are crucial. Assessments of the health risks and mutagenic potential of natural and synthetic substances must consider the long incubation times of some protein-folding diseases, potential connections between protein-folding and other epigenetic phenomena, and the inherently stochastic nature of protein folding itself.

In decades past, biochemists thought the primary sequence of a protein alone determined its functional tertiary structure. Yet it is now acknowledged that, in some cases, a single primary sequence may produce multiple functional structures with different phenotypic consequences.[108] Similarly, the genome is not a deterministic blueprint for an organism's phenotype; a myriad influences shape the translation of genotype to phenotype. When assessing phenotype and analyzing phenotypic evolution, one must look beyond genotype to the vast, as yet not well understood, complexity and interactions of protein-based processes, both within and between cells.

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