Molecular mechanisms of canalization: Hsp90 and beyond

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The Hsp90 chaperone machine facilitates the maturation of a diverse set of 'client' proteins. Many of these Hsp90 clients are essential nodes in signal transduction pathways and regulatory circuits, accounting for the important role Hsp90 plays in organismal development and responses to the environment. Recent findings suggest a broader impact of the chaperone on phenotype: fully functional Hsp90 canalizes wild-type phenotypes by suppressing underlying genetic and epigenetic variation. This variation can be expressed upon challenging the Hsp90 machinery by environmental stress, genetic or pharmaceutical targeting of Hsp90. The existence of Hsp90-buffered genetic and epigenetic variation together with plausible release mechanisms has wide-ranging implication for phenotype and possibly evolutionary processes. Here, we discuss the role of Hsp90 in canalization and organismal plasticity, and highlight important questions for future experimental inquiry.

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1. Introduction

The chaperone Hsp90 is an abundant, heat-induced, essential protein in all eukaryotes studied thus far. The high sequence conservation of Hsp90 proteins across eukaryotes suggests that its functions may also be conserved across species. Indeed, when the mammalian glucocorticoid receptor is introduced into yeast or plants lacking the orthologous protein, the mammalian protein folds and acquires its proper function via its well-studied interaction with the respective endogenous Hsp90 machinery (Picard *et al* 1990). When Hsp90 function is inhibited, client proteins of diverse origins and cellular function typically become unstable and are degraded rapidly. Based on several case examples, Hsp90 is thought to stabilize its client proteins in a signal-competent state. Importantly, Hsp90 dependence can be acquired through mutation and varies dramatically between closely related proteins, presumably due to subtle structural differences (Nathan *et al* 1997; Citri *et al* 2002).

In plants, Hsp90 function remained little characterized until recent studies identified R-proteins as Hsp90 clients (Hubert *et al* 2003; Lu *et al* 2003; Liu *et al* 2004; Sangster and Queitsch 2005). R-proteins function in plant defense against microbial pathogens. The activation of R-protein mediated defense by pathogen-specific effector molecules results in local cell death to limit pathogen proliferation in addition to generating systemic defense signals. In order to avoid unwarranted tissue damage, R-protein activation is tightly controlled, and Hsp90 appears to be involved in fine-tuning R-protein stabilization and activation (Hubert *et al* 2003; Lu *et al* 2003; Liu *et al* 2004; Sangster and Queitsch 2005). In addition to facilitating defense against pathogens, Hsp90 plays a pivotal role in other classic plastic responses such as light perception, seedling etiolation, and gravitropism (Cao *et al* 2000; Queitsch *et al* 2002; Hubert *et al* 2003). Moreover, recent studies report the production of highly specific Hsp90 inhibitors by fungi living in rhizospheres of diverse plant species, establishing Hsp90 as a potential target in interactions between organisms (Gomes *et al* 2003; Turbyville *et al* 2006).

Several recent reports identified Hsp90 as an evolutionarily conserved molecular mechanism affecting phenotypic variance (Rutherford and Lindquist 1998; Queitsch *et al* 2002; Sollars *et al* 2003), which would provide the raw material for natural selection if such variance had a genetic basis. In general, wild-type phenotypes are

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surprisingly robust to perturbations arising from genetic, epigenetic, or environmental variation – in other words, they are 'canalized'. However, evolutionary novelty continues to arise suggesting the existence of molecular mechanisms that can disrupt wild-type robustness and rapidly generate selectable phenotypic variation. Remarkably, interference with the function of a single gene, Hsp90, globally disrupted canalization in flies and plants, revealing altered phenotypes in a background-specific manner. Moreover, such Hsp90-dependent phenotypes can be fixed through selection. Both genetic and epigenetic mechanisms for the fixation of Hsp90-buffered polymorphisms have been proposed (Rutherford and Lindquist 1998; Queitsch *et al* 2002; Sollars *et al* 2003), however, neither Hsp90-buffered genetic variants nor the molecular underpinnings of Hsp90-dependent epigenetic phenotypes are yet identified. Future experiments will have to address the interplay of both genetics and epigenetics and determine the relevance of Hsp90-mediated buffering in nature. To date, the chaperone Hsp90 is the only described molecule with a canalization function; however, newly emerging evidence highlights the canalization potential of other highly connected cellular nodes or other molecular mechanisms (Bergman and Siegal 2003; Raser and O'Shea 2005; Arias and Hayward 2006; Lehner *et al* 2006).

2. Hsp90 and canalization – Uncovering of genetic and epigenetic variation

Over sixty years ago, CH Waddington noted: "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." He named this phenomenon "canalization". As Waddington and his colleagues, however, were able to show, once the proposed buffering capacity is exhausted by a sufficiently severe perturbation, altered phenotypes can arise in diverse organisms (Waddington 1953, 1956; McLaren 1999). Surprisingly, some of these environmentally induced phenotypes were heritable. Successive breeding of affected individuals increased phenotype frequency in the population to near fixation. Importantly, these fixed phenotypes were no longer environmentally sensitive; they had become a canalized, robust trait (genetic assimilation i.e. fixation of environmentally induced phenotypes) (Waddington 1953, 1956). The existence of canalization and the apparent inheritance of such environmentally induced, "acquired" characters were and are controversial.

In their seminal 1998 study, Suzannah Rutherford and Susan Lindquist identified Hsp90 as one possible molecular mechanism for canalization and genetic assimilation (Rutherford and Lindquist 1998). As a highly connected node in many genetic circuits, the environmentally responsive chaperone Hsp90 is an ideal candidate for Waddington's canalization, as it may buffer both environmental and genetic perturbations. Upon interference with Hsp90 function by either mutation or pharmaceutical means, a large spectrum of altered fly phenotypes were expressed, affecting every possible morphological feature in a strain-specific manner. First, Rutherford and Lindquist firmly established that these novel phenotypes were due to reduced Hsp90 function. Second, they showed that an Hsp90-dependent eye and a wing trait, the latter reminiscent of Waddington's heat stress-induced wing phenotypes, were brought to near fixation through breeding of affected individuals. Third, temperature change alone could produce the Hsp90-dependent phenotypes in a strain-specific manner. Importantly, just as Waddington had observed 50 years earlier for his environmentally induced traits (Waddington 1953, 1956), after selection, the fixed altered phenotypes were environmentally robust i.e. they were no longer dependent on Hsp90 inhibition or responsive to temperature.

The concept of Hsp90 as a possible capacitor for morphological evolution has been intensely debated. Critics have argued that the novel fly phenotypes often affected the body plan unilaterally and were likely to decrease fitness severely. Moreover, concerns were raised that an "evolvability" mechanism providing future benefits cannot arise by natural selection that acts on present phenotypes (Dickinson and Seger 1999; Meiklejohn and Hartl 2002). The latter argument can be readily refuted since Hsp90's observed buffering function is most likely a byproduct of its essential biochemical role in chaperoning metastable signal transduction proteins.

The question about the global role of Hsp90 as a canalization mechanism, however, remained. By demonstrating Hsp90-dependent buffering of genetic variation in the plant *A. thaliana*, Queitsch *et al* proved the conservation of this phenomenon between the animal and plant kingdoms (Queitsch *et al* 2002). This study extended the concept of Hsp90 buffering of morphological traits to environmental response pathways by investigating traits such as seedling etiolation and gravitropism. Manipulation of Hsp90 function significantly altered the pattern of natural variation in these classic plastic traits and nearly abolished plasticity in some genetic backgrounds. Unlike partially penetrant morphological fly or plant traits, values for these phenotypes were readily and unambiguously measurable, readily allowing the application of quantitative genetics to identify natural Hsp90-buffered genetic polymorphisms. In plants, reduction of Hsp90 function also resulted in a dramatic increase in phenotypic variation in the absence of genetic variation possibly due to decreased developmental stability or epigenetic phenomena (figure 1). This dual influence of Hsp90 on developmental stability and genetic variation is consistent with Waddington's concept and later theoretical and empirical studies on canalization

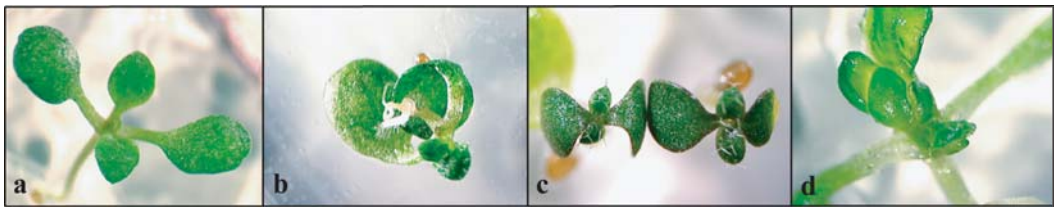

Figure 1. Hsp90 manipulation by geldanamycin drug treatment affects phenotypic variance depending upon genotype in isogenic populations. (**a**) untreated 14-day-old L*er* seedling. (**b, c, d**) seedlings with altered phenotypes due to Hsp90 inhibition. (**b**) common L*er* phenotype. (**c**) common Col phenotype. (**d**) Col/L*er* F_1 seedling. Figure adapted from Queitsch *et al* (2002).

(Waddington 1942; Waddington 1953; Meiklejohn and Hartl 2002; Milton *et al* 2003; Milton *et al* 2006).

Recent studies in *D. melanogaster* added another facet to Hsp90's role in buffering phenotypic variation. Sollars *et al* demonstrated that in some cases epigenetic inheritance rather than genetic inheritance can lead to the fixation of initially Hsp90-dependent morphological traits (Sollars *et al* 2003). An enhancer screen with flies carrying the dominant gain-of function mutation, *krüppel,* identified several mutations that produced ectopic eye outgrowth and extra bristles when maternally inherited. Most of these mutations were either Hsp90 alleles or mutants in trithorax genes known to affect chromatin remodeling. Ectopic outgrowth was also observed when highly inbred flies with the *Krüppel* polymorphism were fed with a diet containing a pharmacological inhibitor of Hsp90. Remarkably, once established, the novel trait increased in penetrance through successive selection in the absence of further Hsp90 impairment and underlying genetic variation. Ectopic outgrowth phenotypes could be reversed by treatment with histone deacetylase (HDAC) inhibitors, consistent with an epigenetic genesis of these phenotypes. Note, however, that the observed phenomenon is not entirely epigenetically determined, as it depends on the presence of the predisposing *krüppel* mutation. It remains unknown if Hsp90 and chromatin remodeling factors act independently or in concert. The possibility of concerted action is supported by a recent study in *Saccharomyces cerevisiae,* which investigated Hsp90's interactions with all possible yeast proteins. Notably, a molecular link of yeast Hsp90 with important components of chromatin remodeling was identified (Zhao *et al* 2005). Further inquiry into the respective contributions of genetics versus epigenetics as well as the identity and frequency of Hsp90-buffered genetic polymorphisms will open many avenues for further experimental exploration of the chaperone's suggested role in evolutionary processes.

3. Hsp90 and plasticity

Plasticity describes the phenomenon that a given genotype can result in different distinct phenotypes depending on its environmental settings. The often dramatic effects of growth conditions including temperature on phenotype in diverse organisms have been widely reported (Durrant 1962; Conover and Kynard 1981; Cullis *et al* 1999; Chen *et al* 2005; Werner *et al* 2005). Plastic responses can be highly regulated and adaptive such as the aforementioned seedling etiolation (Schmitt *et al* 1999; Maloof *et al* 2001) or induced defense against enemies (Baldwin 1998).

In the dark, *A. thaliana* seedlings extend their hypocotyls (seedling etiolation), whereas in the light, green cotyledons expand and leaves develop. Many of the molecular components of the tremendously complex genetic circuit that underlies this deceivingly simple developmental decision have been identified (Chen *et al* 2004). Seedling etiolation is highly sensitive to Hsp90, resulting in near abolition of plasticity for some genetic backgrounds with their hypocotyl length in the dark resembling hypocotyl length in the light, indicating the presence of Hsp90-sensitive genetic variation in this signal transduction network. In plants, Hsp90 appears to be implicated in several environmental response pathways. For example, *CR88*, a chloroplast isoform of Hsp90 (Hsp90.5), is epistatic to the major red light sensing photoreceptor in plants, *phytochrome B,* which mediates shade avoidance (Cao *et al* 2000; Cao *et al* 2003). Furthermore, numerous studies have demonstrated that Hsp90 is important for the stability of multiple R proteins in diverse plant species and that reduction of Hsp90 function results in higher sensitivity to various pathogens (Hubert *et al* 2003; Lu *et al* 2003; Liu *et al* 2004), involving the chaperone in yet another classic induced response. Moreover, our preliminary data indicate that the Hsp90 mutant that is sensitive to microbial pathogens (Hubert *et al* 2003) may be more resistant to generalist herbivores than wild-type. In other words, these Hsp90 mutant plants may be locked in a "non-plastic" state, unable to induce proper defense to pathogens and possibly constitutively up-regulated for defense against herbivores.

Hsp90's effect on plasticity is not limited to plants. In *D. melanogaster,* Hsp90 has been shown to affect the plasticity of developmental traits. For example, flies predisposed to the deformed eye trait expressed dramatically higher

penetrance of this Hsp90-dependent trait with increasing temperature (Rutherford and Lindquist 1998). Note, however, that selection for the deformed eye trait reduced the environmental sensitivity of the trait dramatically. A similar phenomenon was recently reported for a temperature-sensitive polyphenism affecting larval coat color of the tobacco hornworm, *Manduca sexta* (Suzuki and Nijhout 2006). Heat shock during larval development can revert larval coat color of some *M. sexta* carrying the *black* coat color mutation to wild-type green. The authors selected for a line with increased greenness upon heat shock (increased response to heat shock, polyphenic) and a line with decreased color change (unresponsive to heat shock, monophenic) and compared both to unselected controls. Temperature response was markedly increased for the polyphenic line. In contrast, no such response was observed in the monophenic line, black coat color was observed for all animals at all temperatures. This loss of environmentally responsiveness harkens back to Waddington's and Rutherford's selection studies. In summary, an initially environmentally responsive trait may become much less responsive through selection, i.e. it can become canalized through enrichment of genetic variants, new network connections, or epigenetic mechanisms.

4. Hsp90 and evolvability – Facilitating drug resistance in yeast

Although no evidence has been reported for Hsp90-buffering of preexisting genetic variation in *S. cerevisiae,* a recent publication demonstrated an equally important phenomenon in yeast: the Hsp90-dependent rise of new mutations. Specifically, Cowen and Lindquist (2005) found that Hsp90 was essential for the acquisition of mutations resulting in resistance to anti-fungal agents (azoles). The authors succeeded in elucidating the molecular mechanism underlying the Hsp90 sensitivity of acquired azole drug resistance: the alternative pathway typically mediating resistance to azoles requires proper function of the Hsp90 client protein Calcineurin. Remarkably, Hsp90 affected the evolution of drug resistance in different ways in *Candida albicans* and *Aspergillus terreus*, fungi separated from *S. cerevisiae* by millions of years of evolution. Beyond identifying a phenomenon of great medical importance, these findings indicate the possibly broad importance of Hsp90 function in the acquisition of new mutations.

Let us consider both Hsp90-dependent phenomena, uncovering of cryptic genetic variation and acquisitions of new mutations, from a network prospective for genetic circuits. A reduction of Hsp90 may reduce network connectivity and size as certain pathways cease to work for example due to the failure of an Hsp90 client like Calcineurin to fold properly. A reduction in network size and connectivity also decreases the likelihood of new mutations due to diminished sampling space. Similarly, reduced connectivity and network size will reveal cryptic genetic variation in the remaining circuit links that are no longer buffered by the original canalized network. Future experiments will have to determine if the effects of Hsp90 on cryptic genetic variation and the acquisition of new mutations are due to Hsp90 acting on particular client proteins or due to the network properties described here.

5. Is genetic buffering and canalization of development unique to Hsp90?

Certainly not. We have hypothesized previously that any highly connected node can buffer genetic variation and developmental stability (Sangster *et al* 2004). Indeed, other highly connected chaperones such as Hsp70 (which works in concert with Hsp90) and Hsp60 can act in similar fashion in other organisms (Roberts and Feder 1999; Fares *et al* 2002). Moreover, several recent empirical studies and review articles identified candidate genes and molecular mechanisms for developmental canalization (Wagner 2000; Bergman and Siegal 2003; Raser and O'Shea 2005; Arias and Hayward 2006; Hornstein and Shomron 2006; Lehner *et al* 2006). Of particular note, Lehner *et al* suggested a role for chromatin states in canalization, after systematically testing more than 65,000 *Caenorhabditis elegans* pairs of genes for interaction effects on vitality and other fitness-related traits (Lehner *et al* 2006). Surprisingly, a subset of six genes, all encoding highly conserved chromatin remodeling proteins, acted as modifiers of more than a quarter of all queried genes. The authors proposed that these highly conserved 'hubs' may act as buffers of genetic variation in signaling processes in many organisms including humans (Lehner *et al* 2006).

Another ancient and highly conserved molecular mechanism has been recently implicated in developmental canalization in both vertebrates and *D. melanogaster:* small non-coding RNAs. Based on empirical examples of miRNA function, Hornstein and Shomron hypothesize that miRNAs can repress leaky transcription through coordinated action with repressors in so-called coherent feed-forward loops (FFL) (Mangan and Alon 2003; Stark *et al* 2005; Hornstein and Shomron 2006, figure 2A). Similar signal reinforcement is also observed in tissue differentiation where mutually exclusive miRNAs and targets are expressed in cells of opposite developmental fate (figure 2A). miRNAs may also contribute to reduction of noise in gene expression through incoherent FFL (Mangan and Alon 2003; O'Donnell *et al* 2005; Hornstein and Shomron 2006). Here, a miRNA counteracts a transcription factor or repressor, thereby creating a balance of expression which is relatively insensitive to sudden spikes (figure 2B).

Therefore, by ensuring the correct temporal and spatial expression of transcripts, miRNAs may play a very important

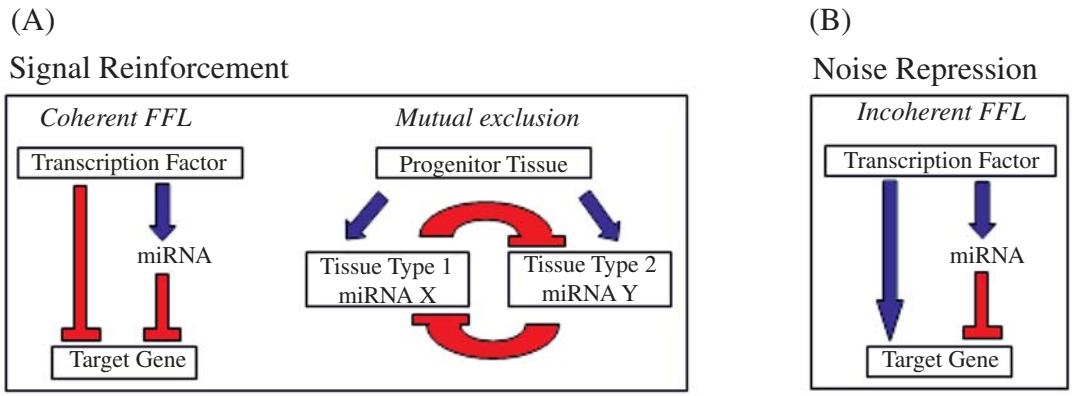

Figure 2. Canalization of developmental pathways by miRNAs. **(A)** miRNAs can prevent 'leaky transcription' through coherent FFLs and sharpen developmental transitions through mutual exclusion (Mangan and Alon 2003). *Left*, repression of a target gene is reinforced by induction of a miRNA targeting the same gene. *Right*, miRNA X is highly produced in tissue type 1 and targets tissue type 2 genes, which are enriched for miRNA X targets. Similarly, miRNA Y is highly produced in tissue type 2 and targets tissue type 1 genes which are enriched for miRNA Y targets. **(B)** miRNAs can repress noise through an incoherent FFL (Mangan and Alon 2003). A transcription factor activates a target gene and a miRNA which represses the same gene. Figure adapted from Hornstein and Shomron, 2006.

role in the canalization of developmental pathways (reviewed by Hornstein and Shomron 2006). Beyond individual developmental pathways and phenotypes, small RNAs may have a much broader role in phenotypic robustness and variation. Recent studies implicate components of the RNAi machinery such as the Argonaute proteins and small RNAs in the formation of chromatin insulators (Lei and Corces 2006) and the integrity of repeated heterochromatic DNA in the nucleolus and elsewhere in the genome (Peng and Karpen 2007). Several leading biologists have referred to small RNAs as the dark matter of biology – in the light of these recent data this is certainly no exaggeration (Michalak 2006).

6. Conclusions

Several recent studies have contributed to our growing understanding of the multifaceted role of Hsp90 in shaping phenotypes during development and in response to many environmental stimuli. As discussed, however, many questions remain unanswered. For example, further empirical studies will have to address the proposed role and importance of Hsp90 in epigenetic processes (Zhao *et al* 2005), which could possibly expand our current view of Hsp90 as a chaperone of mostly cytoplasmic proteins. Similarly, the existence and abundance of highly specific Hsp90 inhibitors in nature (Gomes *et al* 2003; Turbyville *et al* 2006), beckons the systematic exploration of Hsp90 as an interface between organisms with possibly wide-ranging ecological implications.

The molecular underpinnings and parameters of Hsp90's much discussed and reviewed function in buffering genetic variation and developmental stability have remained elusive. Future empirical studies will undoubtedly shed light on the identity, frequency, and fitness effects of buffered polymorphisms in natural populations. In particular, the identity of Hsp90-buffered polymorphisms will illuminate if buffering is predominately due to a direct interaction of Hsp90 with the polymorphism-containing protein or if evidence for the hypothesized network model of buffering can be found (Sangster *et al* 2004). The identity of genes containing Hsp90-buffered polymorphisms will also allow investigating if such genes are enriched for certain molecular functions (e.g., kinases or transcription factors) or biological pathways (e.g., patterning, light signaling, or defense). More importantly, subsequent analysis can then determine if such genes are typically under positive, negative, balancing or neutral selection, thereby directly addressing the role of Hsp90 in evolutionary processes. Finally, the interplay between genetic and epigenetic phenomena and their relative contributions to Hsp90-dependent heritable phenotypes is another field of inquiry whose results will significantly contribute to deciphering the poorly understood molecular underpinnings of Hsp90 buffering.

A thorough understanding of this particular molecular canalization mechanism will certainly aid our search for other canalization mechanisms and thereby our understanding of general principles guiding the translation from genotype to phenotype. Much future experimental work will have to be devoted to investigate the global implications of the above discussed diverse candidate genes and mechanisms for genetic and environmental buffering. Clearly, organisms have succeeded in integrating multiple canalization mechanisms into robust wild-type phenotypes which can

respond appropriately to environmental perturbations and evolve new shapes and functions over time. Now it is up to us to determine how molecules as diverse as a molecular chaperone, chromatin remodeling proteins, and the RNAi machinery interact coherently to achieve such synergy, a truly fascinating and worthy field of future inquiry.

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