ELSEVIER

Seminars in Cell & Developmental Biology 14 (2003) 301–310

seminars in
CELL & DEVELOPMENTAL
BIOLOGY

www.elsevier.com/locate/semcdb

Waddington's widget: Hsp90 and the inheritance of acquired characters

Douglas M. Ruden [a,*], Mark D. Garfinkel [a], Vincent E. Sollars [b], Xiangyi Lu [a]

[a] *Department of Environmental Health Sciences, University of Alabama at Birmingham, Ryals Public Health Building, 1665 University Boulevard, Birmingham, AL 35294-0022, USA*

[b] *Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107, USA*

Abstract

Conrad Waddington published an influential model for evolution in his 1942 paper, *Canalization of Development and Inheritance of Acquired Characters*. In this classic, albeit controversial, paper, he proposed that an unknown mechanism exists that conceals phenotypic variation until the organism is stressed. Recent studies have proposed that the highly conserved chaperone Hsp90 could function as a "capacitor," or an "adaptively inducible canalizer," that masks silent phenotypic variation of either genetic or epigenetic origin. This review will discuss evidence for, and arguments against, the role of Hsp90 as a capacitor for morphological evolution, and as a key component of what we call "Waddington's widget."

Keywords: Hsp90; Evolution; *Drosophila* development; Capacitors; Morphological development; Canalization

1. Introduction

According to Webster's Online Dictionary, a "widget" is "an unnamed article considered for purposes of a hypothetical example." In 1942, Conrad Waddington (1905–1975) published his classic paper, *Canalization and the Inheritance of Acquired Characters*, in which he argued that an unnamed article regulates phenotypic expression of several, apparently acquired, developmental characters. Waddington cited several examples of apparently acquired characteristics that have useful purposes in adult organisms, but little or no function in fetuses, such as the callosities on the knees of fetal ostriches and the thickening soles of the feet of fetal humans [1]. In Waddington's paper, he proposed the existence of what are now called "adaptively inducible canalizers" (Meiklejohn and Hartl's term [2]), or "evolutionary capacitors" (Rutherford and Lindquist's term [3]), that reveal phenotypic variation in times of stress. Waddington proposed that, when variation is selected in subsequent generations, "assimilation" of the new phenotype occurs so that the phenotype can become expressed even in the absence of stress [1]. Waddington further hypothesized that once a novel phenotype is "assimilated," a period of stabilizing selection can cause the phenotype to be "canalized" [1].

In later studies, which Waddington interpreted as confirming his 1942 hypothetical model explaining the apparent inheritance of acquired characters, he showed evidence that an unnamed article affects the *crossveinless* [4] and *Ubx* phenotypes [5] in *Drosophila*. When the unnamed article was altered by stress (heat shock or ether exposure), selection of the exposed phenotype occurred, and, after several generations of selection, the phenotype was "assimilated," i.e. expressed even in the absence of stress. Waddington's experiments beautifully demonstrated "assimilation," but did not adequately address the "canalization" aspect of his model. Rendel, whose work is less cited than Waddington, did experiments on the *scute* bristle phenotype in *Drosophila* that better support the "canalization" aspect of Waddington's model [6,7]. Unfortunately, few scientists paid attention to Waddington and Rendel at the time because their scientific programs were suspect, presumably because they appeared Lamarckian [8]. We will discuss this issue in more detail later, but it is fair to say that Waddington's and Rendel's bodies of work have recently undergone a resurgence of interest.

Because of the mysteriousness of the unnamed articles that either hide or expose phenotypic variation, we refer collectively to the unnamed articles as "Waddington's widget." The molecular mechanism of Waddington's widget remained a mystery until 1998, when Rutherford and Lindquist presented evidence that Hsp90 fulfills the requirements for being a likely component [3]. In this paper, and in a similar study using *Arabidopsis* [9], Lindquist and coworkers showed that genetic or pharmacological inactivation

* Corresponding author. Tel.: +1-205-934-7042; fax: +1-205-934-7142.
E-mail addresses: douglasr@uab.edu (D.M. Ruden), garfinkl@uab.edu (M.D. Garfinkel), xlu@uab.edu (X. Lu).

1084-9521/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.semcdb.2003.09.024

of Hsp90 exposed previously hidden phenotypic variation, and that this phenotypic variation can be selected and eventually assimilated in the population [3].

In apparent contrast to the papers from the Lindquist laboratory, work from our laboratory provided unique evidence for an epigenetic mechanism for the capacitor function of Hsp90 [10]. Recently, several reviews have pondered the possible genetic [11–16] and epigenetic [17–19] roles of Hsp90 in morphological development and evolution. The latter three reviews argue that both genetic and epigenetic mechanisms likely explain the evolutionary capacitor function of Hsp90. In this review, we attempt an unbiased summary of the evidence in favor and the arguments against the proposed capacitor function of Hsp90, and other possible uses of Hsp90 in development and evolution. Foremost, after a short historical perspective on potential mechanisms of evolution, we address the question, "Is Hsp90 Waddington's widget?"

2. Inheritance of acquired characters—an abridged historical perspective

Jean Baptiste Pierre Antoine de Monet, Chevalier de Lamarck (1744–1829) was an influential French naturalist and evolutionary theorist. Lamarck proposed a theory of evolution in his book *Zoological Philosophy* (1809) that maintains that animals acquire useful characteristics during their lifetimes, and that they can pass on these acquired characteristics to their offspring [20]. In this controversial book, contentious even at the time it was first published, Lamarck stated, "continued use of any organ leads to its development, strengthens it and even enlarges it, while permanent disuse of any organ is injurious to its development, causes it to deteriorate, and ultimately disappear if the disuse continues for a long period through generations" [20]. Lamarckian theorists of the 19th Century, known at the time as "naturalists," famously maintained, for instance, that a giraffe first develops a long neck by "striving" to reach tall trees, then passes this characteristic to its young [21].

The Lamarckian theory of inheritance of acquired characteristics was replaced, at least for the majority of 20th Century evolutionary biologists and geneticists, by Charles Darwin's (1809–1882) theory of natural selection that he first published in *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life* (1859) [22]. The main reason, of course, for the continued popularity of Darwin's theory of natural selection in the 20th Century was that a mechanism for selection of discrete units or genes was provided in the laws of genetics proposed by Gregor Mendel (1822–1884) in *Experiments in Plant Hybridization* (1865) [23].

Thomas Hunt Morgan (1866–1945), with his small group at Columbia University, including A.H. Sturtevant (1891–1970), C.B. Bridges (1889–1938), and H.J. Muller (1890–1957), further drove the nail in the coffin of Lamarckism with their pioneering genetics research with *Drosophila*, starting in 1908 [24]. In 1915, Morgan, Bridges, and Sturtevant published *The Mechanism of Mendelian Heredity*, a book that established *Drosophila* as an excellent model system in genetics [25]. In 1928, with the exception of Muller, Morgan moved his group to Caltech where they spent the remainder of their careers [24]. Ironically, before moving to Columbia University and beginning his research with *Drosophila*, Morgan was skeptical of Darwinism, which he perceived to be "too speculative and not grounded in observable phenomena" [24]. Also, at this time early in his career, as was the fashion among many well-respected developmental biologists, Morgan was critical of Mendelism and the chromosomal theory of heredity [24].

Despite the disrepute the majority of 20th Century biologists held (and still hold) for the idea of Lamarckian evolution, T.D. Lysenko (1889–1976), who became an influential agronomist in the Soviet Union during the Stalin years, was a firm adherent [26]. Lysenko discovered that the germination characteristics of winter wheat could be made to mimic spring wheat by the Lamarckian-appearing practice of exposing seeds to moisture and cold. His simple process of "vernalizing" wheat held out the prospect, misplaced as it turned out, of improving wheat yields in the harsh weather of Siberia. Lysenko's experiments were poorly controlled and never subjected to peer review, but a political establishment that viewed environmental malleability of biological attributes as consonant with Marxist ideology accepted them nonetheless.

Large-scale application of Lysenko's practices, combined with the brutal collectivization of agriculture ordered by Stalin in the early 1930s, contributed to severe famines that killed an estimated 10 million Russians. Despite this abject failure, Lysenko was elevated in 1937 to membership in the Supreme Soviet and he became the head of the Institute of Genetics of the Soviet Academy of Sciences. In 1948 he delivered an impassioned address denouncing Mendelian thought as "reactionary and decadent" and declared such thinkers to be "enemies of the Soviet people" [26]. It was due to Lysenko's efforts that many scientists, those who were geneticists or who rejected Lamarckism in favor of natural selection, were imprisoned, executed or exiled [26]. With Stalin's death in 1953, Lysenko's influence began to fade, although a complete repudiation did not occur in the Soviet Union until well over another decade had passed.

The debacle that was Lysenkoism irreparably tarnished the image of Lamarckian evolution for a great majority of scientists. Yet vernalization is a real botanical phenomenon that, ironically, eventually proved tractable to Mendelian genetic experimentation. Studies in the last decade have identified the mechanisms by which photoperiod and exposure to cold regulate the timing of flowering in *Arabidopsis*, wheat, oats and barley, among other plant species. The processes governing cold-stress response and cold tolerance are much less well understood than those involved in oxidative stress

or heat shock, but make use of signal transduction cascades involving gibberellins, MADS-family transcription factors, lectins, and, interestingly, epigenetic modification of the plant genome by DNA methylation (reviewed in [27]).

3. Waddington and 20th Century biology

Early in the 20th Century, before the calamitous Lysenkoism events described above, Lamarckian evolution was still in vogue, at least by the "naturalists" who believed in the inheritance of acquired characters. The "naturalists" fought pitched battles with the "geneticists" who, using Darwinian principles, believed in the inheritance of genetic variants by means of natural selection. In 1942, Waddington tried to mediate the battle between the "naturalists," who were beginning to wane in influence, and the "geneticists" [1]. In his classic paper, he proposed a genetic mechanism for the apparent, but some people argue not genuine (see below), inheritance of acquired characters. According to Waddington, "Once the developmental path has been canalized, it is to be expected that many different agents, including a number of mutations available in the germplasm of the species, will be able to switch development into it. By such a series of steps, then, it is possible that an adaptive response can be fixed without waiting for the occurrence of a mutation" [1]. According to Waddington, canalization is mediated by "Developmental reactions [that] are adjusted so as to bring about one definite end-result regardless of minor variations in conditions during the course of the reaction" [1].

In evolutionary biology, the concept of canalization only became important after Waddington demonstrated genetic assimilation of environmentally induced phenotypes [4,5]. For example, Waddington showed that a *crossveinless* phenotype is induced in a small percentage of offspring when parental *Drosophila* are heat shocked, and that selection of progeny with this environmentally induced phenotype for several generations leads to assimilation of this phenotype in nearly 100% of the progeny, even in the absence of heat shock [4]. Waddington performed similar experiments with the *Ultrabithorax* (*Ubx*) phenotype induced by ether, a *Ubx* "phenocopy" in the jargon of the geneticists, and found that this phenotype can also be assimilated into nearly 100% of the selected population [5]. Subsequently, assimilation experiments were also performed in several laboratories on the *extra cross-veins* phenotype, the *dumpy* larval phenotype, and the *large anal papillae* phenotype in wild-type strains of *Drosophila* (reviewed in [28]).

Another approach that several laboratories have used in assimilation experiments is to select for extreme phenotypes in an already mutant background. For example, wing vein length was selected in ci^D mutant flies, and vibrissae number was selected in *Ta* mutant mice. Again, fixation of an extreme phenotype of either mutation occurs after 10–20 generations of selection. Other examples include selection of facet numbers in *Bar*-mutant *Drosophila*, wing-vein interruptions in *Hairless*-mutant *Drosophila*, and bristle number in *ocelli-less* mutant *Drosophila* (reviewed in [28]).

A.J. Bateman, a graduate student of Waddington, proposed three models to explain the increase in frequency of the *dumpy* phenotype: (1) a shift in the mean of the distribution; (2) a shift of the threshold; or (3) an increase of its variance [29]. She interpreted her data as supporting Model 2, that selection of the *dumpy* phenotype shifts the threshold such that the new genetic makeup of the selected population favors this phenotype [29]. J.M. Rendel did experiments that definitively distinguished between Bateman's models [6,7]. In the phenotype that he studied, the *scute* bristle phenotype, the mutation produced a combination of Models 1 and 3, that is, it shifted the mean phenotype out of the range of a canalizing system, which also resulted in an increase in variance. By selecting on his populations, he showed that the canalizing system was still in place, even in mutant flies when they were brought back to the wild-type mean. In other words, the *scute* mutation moved development out of the range of the canalizing system, without disrupting the system itself [6,7]. In a later section, we incorporate Bateman's three models to explain epigenetic canalization.

According to Scott Gilbert in his popular textbook *Developmental Biology*, "Waddington's work was misinterpreted as supporting the inheritance of acquired traits" [8]. Gilbert's view is surprising because Waddington himself used the phrase "inheritance of acquired characters" in the title of his classic paper described above [1]. Nevertheless, Gilbert espoused this argument because, "While Waddington's results look like a case of 'inheritance of acquired characteristics,' there is no evidence for that view. Certainly, the *crossveinless* phenotype was not an adaptive response to heat. Nor did heat shock cause the mutations. Rather, the heat shock overcame the buffering systems, allowing preexisting mutations to result in mutant phenotypes rather than wild-type phenotypes." Gilbert was evidently striving to revitalize Waddington's reputation by distancing Waddington's ideas from Lamarckian evolution. As discussed below, perhaps this was not necessary.

4. Hsp90 as a capacitor for morphological evolution

Hsp90 is unique in its functions as a major heat shock protein. Unlike the other proteins in this class of stress-induced proteins, Hsp90 is not required for the maturation or maintenance of proteins in general. Rather, most of the identified cellular targets of Hsp90 are involved in signal transduction and chromatin organization (reviewed in [16,30]). Several cell cycle and developmental regulators have been shown to form non-functional conformations in the absence of Hsp90, and Hsp90 activates these signaling pathways by stabilizing their alternate, functional, conformations [30]. It has been postulated that low-affinity interactions of Hsp90 and its targets keeps the signaling proteins poised for activation until they are activated by post-translational modifications from

upstream signaling molecules [30]. For example, several members of steroid–hormone receptors, cyclin-dependent kinases, and Src-family kinases have been shown to be substrates for Hsp90 [30]. Recently, Hsp90 itself has been shown to be associated with the chromatin in complexes that destabilize the estrogen receptor transcriptional activation complex [31].

Rutherford and Lindquist showed that reduction of Hsp90 reveals previously concealed phenotypic variation in *Drosophila* [3]. They showed that when mutation or pharmacological inhibitors reduced Hsp90 activity, phenotypic variation of seven adult structures of the fly is induced. As Waddington had done with the *crossveinless* phenotype, Rutherford and Lindquist observed that selection for 10 or more generations of a particular adult structural abnormality leads to the assimilation of the new phenotype in the population. Also, as Waddington had seen with the *crossveinless* phenotype induced by heat shock [4], assimilation of the phenotypes in the populations selected by Rutherford and Lindquist occurs after the restoration of normal levels of Hsp90.

Evidence that this is primarily a genetic rather than an epigenetic phenomenon is that adult structures are differentially altered in strain-dependent manners in laboratory strains and wild populations. Also, in what Lindquist and coworkers later called a "litmus test for the genetic basis of traits" [19], they show that outcrossing the selected flies with high trait penetrance to unselected lines heterozygous for the *Hsp90* mutation results in only the *Hsp90*-mutant progeny having the selected phenotype [19]. Their interpretation is that, "Should the trait have been epigenetically inherited, it would not have disappeared in outcrossed progeny with wild-type Hsp90 levels" [19].

Our experiments, described below, did not pass the "litmus test" for genetic traits because we still saw the enhanced $Kr^{If\text{-}1}$ phenotype when we outcrossed selected flies to unselected flies with the same *iso-*$Kr^{If\text{-}1}$ background ([10] and data not shown). However, we can imagine situations where an epigenetically inherited trait disappears in outcrossed progeny. For example, the outcross strain might have a higher than usual concentration of chromatin inhibiting proteins, such as a histone H3 lysine 9 methyltransferase, like Su(var)3–9, that plays a central role in heterochromatic gene silencing [32]. Also, strictly speaking, Lindquist and coworkers [3,9] could not exclude an *additional* contribution due to heritable epigenetic, or chromatin-conformational, variation.

5. Hsp90 is a capacitor for cryptic morphological variation in plants

Levels and patterns of genetic variation differ greatly between outbreeding species such as *Drosophila* and self-fertilizing species such as the plant *Arabidopsis thaliana*. One might speculate that inbreeding species, because of their nearly isogenic genomes, would have much less phenotypic variation, at least within an isolate, than outbreeding species. However, Queitsch et al. show in their recent paper that *Arabidopsis thaliana* isolates can have dramatic phenotypic variation when Hsp90 activity is pharmacologically reduced [9]. As Rutherford and Lindquist showed in *Drosophila* [3], Queitsch et al. showed that reducing Hsp90 function produces an array of morphological phenotypes in *Arabidopsis* accessions and recombinant inbred lines [9]. Interestingly, the induced phenotypic variations are dependent on underlying genetic variation, despite the fact that very little phenotypic variation was present prior to abrogating Hsp90 function and subsequent selection.

Since different *Arabidopsis* isolates develop different phenotypic alterations, in a strain-specific manner, Queitsch et al. argued that the effects of reducing Hsp90 are genetic rather than epigenetic in nature [9]. However, as with Lindquist's and coworkers' *Drosophila* experiments, they did not specifically rule out an additional epigenetic contribution to the effects that they observed [9].

6. Evidence that Hsp90 functions as a capacitor for morphological evolution in an epigenetic manner

We reported evidence that Hsp90 affects development by altering the chromatin in the eye imaginal disc [10]. Our intent was to determine whether Hsp90 could function as a capacitor for morphological development by an epigenetic mechanism in a sensitized system. In 1957, Bateman attempted to perform a canalization experiment with the *crossveinless* phenotype in an isogenized strain [33]. However, her experiment failed, possibly because she did not use, as we did, a sensitized strain [33]. The reason we wanted to test this "chromatin hypothesis" is because we had isolated mutations in both Hsp90 and several Trithorax Group (TrxG) genes as maternal enhancers of the $Kr^{If\text{-}1}$ "developmentally sensitized" eye phenotype (Fig. 1) [10]. The most efficient of the maternal enhancers of the $Kr^{If\text{-}1}$ phenotype was a mutation in the TrxG gene *verthandi* (*vtd*), which showed over 90% expression of an eye-bristle phenotype in the male progeny, and over 10% expression of the phenotype in the female progeny [10]. The other enhancers only had 5–15% expression of the enhanced phenotype in both male and female progeny.

Surprisingly, we found that the enhanced $Kr^{If\text{-}1}$ phenotype was transmitted in several subsequent generations, through both the male and female germlines, even in the absence of the initiator vtd^3 mutation [10]. Also, the penetrance of the phenotype increased in a selection experiment in the absence of the vtd^3 mutation [10]. While we acknowledged that a genetic mechanism could explain this result, the fact that TrxG proteins affect chromatin structure, generally in manners that promote transcription, suggested the likelihood of an epigenetic mechanism [10]. Also, the fact that histone deacetylase inhibitors such as trichostatin A and sodium bu-

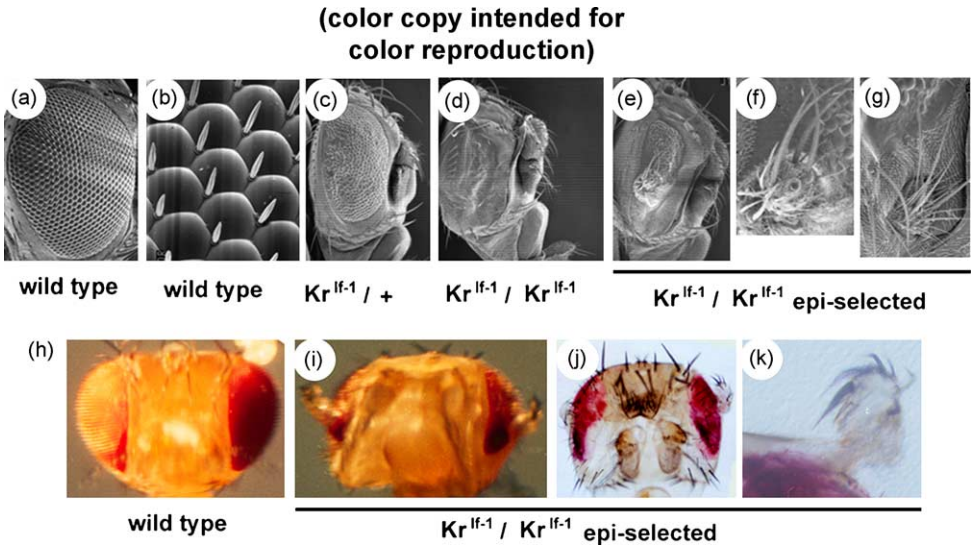

Fig. 1. Examples of the epigenetically-selected $Kr^{If\text{-}1}$ phenotype. Parts (a)–(g) are transmission electon micrographs, (h) and (i) are light micrographs of intact heads, and (j) and (k) are light micrographs of fly heads flatten in Hoyer's mount [10]. The genotypes of the flies are indicated, and examples of the epigenetically selected flies with bristle outgrowths are shown.

tyrate reduced the penetrance of the enhanced $Kr^{If\text{-}1}$ phenotype also supported an epigenetic mechanism [10].

The way that we sought to more rigorously test the epigenetic capacitor hypothesis was by removing, as much as possible, all sources of genetic variation in a sensitized *Drosophila* strain, *iso-*$Kr^{If\text{-}1}$ [10,34]. We enhanced the $Kr^{If\text{-}1}$ phenotype by feeding these isogenized flies the potent and specific Hsp90 inhibitor geldanamycin [10]. The heart of Waddington's assimilation hypothesis is that genetic variation must be present for selection of a novel phenotype after an environmental stress. Nevertheless, we still observed an enhanced $Kr^{If\text{-}1}$ phenotype, whose penetrance increased in a 13-generation selection experiment (Fig. 1) [10].

Further evidence that what we were observing was an epigenetic phenomenon was that the enhanced $Kr^{If\text{-}1}$ phenotype was unstable, even after 13 generations of selection, and that the penetrance was never greater than 70% [10]. However, this lack of complete penetrance is not necessarily a uniquely epigenetic phenomenon because many presumed genetically determined phenotypes under artificial selection also reach a selection plateau that is not the result of an exhaustion of the genetic variation from the selected population, but rather from a balance between natural and artificial selection [28]. That is, the selected individuals might be so sick that any individuals with more extreme phenotypes are sterile or inviable. In these cases, if the artificial selection is relaxed, the population would quickly move back in the direction of the original phenotypic distribution. Such viability issues could produce both the incomplete penetrance and long-term instability that we attribute to epigenetic factors in our system.

The stability of genetically assimilated phenotypes that do not decrease viability or fertility is demonstrated in J. Hirsch's selection of the geotaxis phenotype in *Drosophila* published in 1959 [35]. The high and the low geotaxis strains were selected for over 20 generations, and then propagated for over 40 years without further selection for these phenotypes. Remarkably, the phenotypes were as stable as they were 40 years earlier [36], thus supporting a genetic assimilation mechanism, rather than a transient epigenetic assimilation mechanism.

In studies to date with *iso-*$Kr^{If\text{-}1}$, we did not rule out the existence or importance of cryptic genes, but instead we showed that the existence of cryptic genes is probably not necessary to explain at least some of our observations. We believe that the existence of cryptic genes is also probably not necessary to explain some of the results of Waddington [4,5], Rutherford and Lindquist [3] and Queitsch et al. [9]. For example, Queitsch et al. showed that some of the Hsp90-inhibitor-induced phenotypes in *Arabidopsis* were due to genetic variation, but others could not be propagated in isogenic lines [9]. Because of their instability, it is possible that epigenetic effects caused some of the non-propagatable phenotypes.

While it is true that other laboratories have shown that epigenetic states can be inherited, that the frequency of an epigenetic state within a population can be selected to increase by breeding individuals with these states, and that the propensity for individuals to adopt certain epigenetic states is influenced by that organism's genotype and environment [17], we believe that our contribution to evolutionary theory is that Hsp90 can function as a capacitor for morphological development in an epigenetic manner. Rutherford and Lindquist [3] and Queitsch et al. [9] did not address the possibility that Hsp90 might function in an epigenetic manner. According to Massimo Pigliucci, a prominent evolutionary theorist, "This [Sollars et al.] is one of the most convincing

pieces of evidence that epigenetic variation is far from being a curious nuisance to evolutionary biologists, but may play a fundamental role in adaptation to rapidly changing environmental conditions, side by side with standard genetic variation" [17].

7. Models for the epigenetic function of Hsp90

Sangster et al. recently proposed a speculative model for how *vtd*^{3}, the most potent enhancer of *Kr*$^{If\text{-}1}$, enhances the *Kr*$^{If\text{-}1}$ phenotype [19]. These authors propose, since *vtd* maps to a region near the centromere of chromosome 3, a region with few unique DNA sequences, that *vtd* is not a gene that encodes a protein, but rather a chromatin regulatory locus [19]. One of the few protein-encoding genes in the region containing *vtd* is the *alpha-catenin* gene, a key component of the Wingless-signaling pathway [19]. They hypothesized that the *vtd*^{3} mutation causes a "spread in the nearby heterochromatin" to the *alpha-catenin* gene, and that this inactivation is heritable, "if one invokes that heterochromatic spread due to loss of one *vtd* element may be transmitted to an intact homolog by a *trans*-silencing mechanism" [19]. This model is consistent with our original proposal that ectopic Wg signaling was one of the epigenetic causes of the enhanced *Kr*$^{If\text{-}1}$ phenotype ([10], Fig. 2, and additional data not shown). However, more recent analyses indicate that what we observed is probably not ectopic Wg expression in the peripodial membrane, but rather ectopic adhesion of hemocytes under the disc (Fig. 2). This makes sense because hemocytes are involved in tissue remodeling,

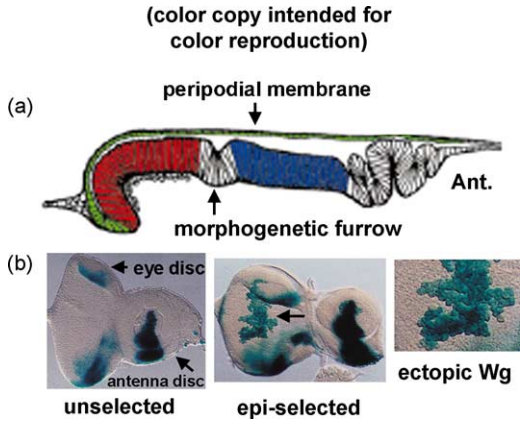

Fig. 2. Reduction of Hsp90 activity allows multi-generational epigenetic inheritance of ectopic hemocyte adhesion. (a) Schematic diagram of an eye imaginal disc. The morphogenetic furrow that moves from the posterior to the anterior (Ant.) is shown. The differentiated ommatidia are indicated in red, and the undifferentiated columnal epithelial cells of the disc proper are indicated in blue (adapted from [55]). (b) Left, shows a third instar eye and antennal imaginal disc expressing *wg*^{02657}-*lacZ* (blue). Middle, shows ectopic hemocyte adhesion from the progeny of epigenetically selected *iso-Kr*^{If} female flies mated to *wg*^{02657}-*lacZ* males [10]. Right, shows a higher magnification of the ectopic *wg*^{02657}-*lacZ* expression from the middle panel.

such as presumably occurs when eyes are induced to have ectopic outgrowths.

However, we feel that the model of Sangster et al. [19] highly speculative. For instance, attempts to identify the *vtd* gene in the sequenced *Drosophila* genome might have failed because *vtd* could have small exons interspersed over a large region of heterochromatin. There are examples of genes on the Y-chromosome that resist annotation attempts because they are very large, and have small exons interspersed in large blocks of heterochromatic regions [37]. Also, while reduction of *alpha-catenin* expression via a heterochromatic mechanism would be expected to increase the efficacy of Wg signaling, it would probably not increase the amount of Wg protein itself. Furthermore, Wg expression is not in a positive-feedback loop in any of the known Wg signaling pathways [38].

We prefer a model in which the chromatin of Kr target genes in the columnal epithelial cells is sensitized for activation or repression by a reduction in Hsp90 protein levels. It is also possible that expression of signaling molecules other than Wg is altered by a reduction in Hsp90. Of possible relevance is the finding from Schubiger's laboratory that stress, caused by cutting off pieces of wing imaginal discs, activates Hh expression in the peripodial membrane of second instar larval discs [39,40]. Ectopic Hh expression in second instar larval discs could be causing the ectopic hemocyte adhesion that we observe in the peripodial membranes of *vtd*^{3}-mutant third instar larval eye discs ([10]; Fig. 2), but this has not yet been tested. It is also possible that the different maternal enhancers of *Kr*^{If}, such as *vtd* and *Hsp90* mutations, could be functioning through independent chromatin-regulatory mechanisms.

In Fig. 3, we present three, non-mutually exclusive, models for *epigenetic* assimilation of a new phenotype by Hsp90. These models are similar to those proposed by Bateman, described above, to explain the *genetic* assimilation of new phenotypes—(1) a shift in the mean, (2) a shift in the threshold, and (3) an increase in the variance of the phenotype [29]. In a review article discussing our paper, Rutherford and Henikoff propose Model 1, in which the mean for the enhanced *Kr*$^{If\text{-}1}$ phenotype is shifted by a reduction in Hsp90 [18]. As discussed above, Bateman preferred Model 2, a shift in the threshold, for her *genetic* assimilation experiments [29], whereas Rendel's data supports a combination of Models 1 and 3 [6,7]. We are leaning towards Model 3, an increase in the variance of the phenotype, because recent results with lead acetate-fed flies from our laboratory [41], and our collaborators' laboratories [42,43], support this view.

8. Lamarckian evolution revisited

Whereas the papers on Hsp90 by Rutherford and Lindquist [3] and Queitsch et al. [9] reaffirm Gilbert's contention, quoted above, that there is no evidence to support the idea that they were observing "inheritance of acquired

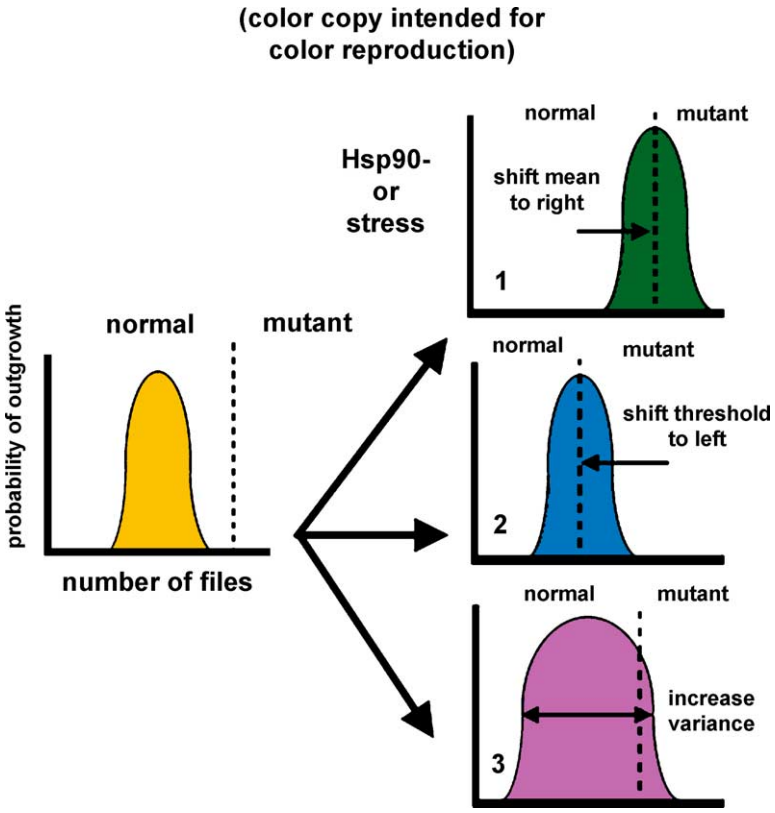

Fig. 3. Three models for the epigenetic function of Hsp90. Model 1 is that selection of the epigenetically induced phenotype shifts the mean of the distribution towards the threshold. Model 2 is that the threshold is shifted towards the original mean. Model 3 is that the variance of the distribution of the phenotype is increased (adapted from [29], see text).

characterstics" [3], we believe that our paper does support this aspect of Lamarckian evolution [10]. Whereas neither the *crossveinless* phenotype observed by Waddington [1], nor the ectopic eye bristles that we observe in $Kr^{If\text{-}1}$ flies are adaptive responses to stress, we believe it is likely that some Hsp90-induced chromatin alterations are an adaptive response to stress. For example, chromatin alterations of the heat shock genes could, at least partly, explain the acute tolerance to stress observed during repeated heat shocks [44,45].

While stress probably does not cause mutations in our epigenetic system, it evidently causes heritable chromatin alterations [10]. We cannot rule out the possibility that geldanamycin-induced stress is causing new mutations in our *iso-*$Kr^{If\text{-}1}$ strain, as stress reportedly causes "adaptive increases in mutation rates" in mutator strains of bacteria [46]. Below, we present a hypothetical combined Lamarckian–Darwinian example for our epigenetic model for evolution. However, without further evidence for the generality of epigenetic effects on evolution, one may take such models, as suggested by Pigliucci, with "a grain of salt" [17].

In our hypothetical example, an animal species transported to a new environment could undergo starvation stress, for instance, and this type of stress would cause a decrease in Hsp90, and all other proteins for that matter, because of a limited amino acid pool [47]. A reduction in Hsp90 levels would likely lead to chromatin changes, as we observe in *Drosophila* [10], and the chromatin changes could potentially cause a range of random morphological phenotypes in the progeny. The progeny with adaptive morphological variation, such as long necks in the Lamarckian giraffe example [21], and long beaks in the Darwinian finch example [22], would be the ones who survive to reproductive age. Finally, and a combination of epigenetic [10] and genetic [3] selection would occur to assimilate the new phenotype in subsequent generations. While we do not agree with many of the concepts of Lamarckian evolution, such as the giraffe "striving" to reach the tops of trees, we do argue that many of them should be revisited.

Thus, our paradigm requires the passing on of acquired characteristics, unquestionably a Lamarckian concept, but importantly, those characteristics are still generated at random with respect to the needs of the organism, a Darwinian concept. In other words, our formulation still produces random epigenetic variation produced by loss of Hsp90, which is sorted by natural selection. Kenneth Weiss more elegantly stated a similar point in his paper discussing the pros and cons of Mendelian genetics [48]. In this paper, he said, "Like the famous princess, we seem to think that we can always detect a Mendelian pea no matter how many layers of environmental and other influences may lie over it" [48].

9. Evolutionary implications of the capacitor function of Hsp90

One criticism of Hsp90 as an "evolutionary capacitor" is that all of the phenotypes are severely deleterious, and it is hard to imagine how individuals that have had these genetic or epigenetic variants revealed would have any advantage over buffered individuals [3,9,10]. For example, Meiklejohn and Hartl state in their review of canalization, "Over evolutionary time, the frequency with which a phenotypically revealed allele provides a selective advantage greater than the negative consequences of removing environmental canalization is likely to be extremely small" [2]. Waddington made a persuasive response to similar criticism in his 1953 paper on the assimilation of the *crossveinless* phenotype [4]. Waddington said, "There is, of course, no reason to believe that the phenocopy (the *crossveinless* phenotype) would in nature have any adaptive value, but the point at issue is whether it would be eventually genetically assimilated if it were favored by selection, as it can be under experimental conditions" [4]. Similarly, there is little reason to believe that the ectopic outgrowths in the eyes of $Kr^{If\text{-}1}$ flies that we observe would in nature have any adaptive value, but the point of the experiments was to determine, as a proof of concept, whether they could be epigenetically assimilated if it were favored by selection.

One could imagine a situation where the ectopic outgrowths have an adaptive value, as is apparently the case with stalk-eyed female flies preferring males having long eye stalks as a form of mate selection [49], or with other *Drosophilidae* with unusually shaped head capsules and eyes [50]. However, Darwin himself treats sexual selection as a special case, and oftentimes an exception, to his "survival of the fittest" model [22].

Gilbert made another argument in favor of evolutionary capacitors in a discussion favoring the introduction of a capacitor model, and developmental biology models in general, into evolutionary theory. Gilbert said, "The developmental genetics approach to evolution concerns more the *arrival* of the fittest than the *survival* of the fittest" [8]. In other words, the capacitor model could explain how stress induces random morphological variation, but further models are needed to determine the mechanism of assimilation of the adaptive morphological changes.

Rutherford and Lindquist proposed that the capacitor function of Hsp90 has been selected during evolution [3]. However, it would seem impossible for a group-level trait such as a morphological capacitor to be selected since it would only benefit distant generations and not the immediate generation. Indeed, the capacitor activity of Hsp90 is likely a perfect example of a "spandrel," a term that Stephen J. Gould (1941–2002) borrowed from architecture to designate "the class of forms and spaces that arise as necessary byproducts of another decision in design, and not as adaptations for direct utility in themselves" [51]. The capacitor function of Hsp90, such as it is, is likely a spandrel of its everyday function as a chaperone for developmentally important signaling molecules.

However, calling the capacitor function of Hsp90 a "spandrel" should not diminish the importance of this activity [2]. Gould points out the evolutionary importance of spandrels by saying, "These sequelae—spandrels in the terminology of this paper—arise nonadaptively as architectural byproducts but may regulate, and even dominate, the later history of a lineage as a result of their capacity for co-optation to subsequent (*and evolutionary crucial*) utility" [52] [emphasis added]. What better protein to be co-opted for an evolutionary crucial utility than a chaperone for numerous signaling pathways, such as Hsp90? Co-optation of a regulator of regulators such as Hsp90 allows the generation of novel structures, such as eye appendages, in a single generation—a feat that cannot be accomplished by the co-optation of any individual signaling molecule.

10. Arguments against the Hsp90 capacitor models

The question of whether the phenotypes revealed by Hsp90 are beneficial or deleterious is the most misunderstood criticism of the capacitor hypothesis. Some critics concede that, upon rare occasion, a phenotype that is revealed by a lack of Hsp90 might be advantageous. However, they argue that the plausibility of the capacitor hypothesis depends critically not only on the existence of beneficial phenotypes being revealed, but also on the relative frequency of beneficial phenotypes as compared with deleterious phenotypes [2]. In discussing these arguments against capacitor models, we will distinguish between *biochemical* phenotypic variation, which can affect the immediate as well as subsequent generations, and *morphological* phenotypic variation, which can only affect subsequent populations because most reproductively mature organisms cannot undergo further morphological changes.

As an example (Colin Meiklejohn, personal communication), suppose that a species that is undergoing repeated bouts of stress has some individuals with a capacitor allele of Hsp90, and some individuals with a non-capacitor allele—i.e. one that is not reduced in activity by stress, and thereby releases cryptic *biochemical* phenotypic variation in the immediate population. There are three possible consequences of having the Hsp90 capacitor allele—(1) no effect, (2) negative effect, and (3) positive effect. Assuming that the majority of the biological consequences caused by reduction of Hsp90 during stress are deleterious, say 90%, then the population with the capacitor version of Hsp90 would have to survive 9 or 10 deleterious effects for every beneficial one. In contrast, the population with the non-capacitor allele would never undergo the deleterious events caused by having too little Hsp90 during the repeated bouts of stress. Critics argue, strictly on theoretical grounds, that the population with the capacitor version of Hsp90 is "less fit" because of the preponderance of exposed deleterious cryptic biochemical and

morphological phenotypic variation and would, over many generations of repeated bouts of stress, eventually become extinct.

We believe that the above criticisms, while logically valid for the original canalization and capacitor hypotheses, are somewhat muted, at least for the inheritance of phenotypic variation over many generations, by incorporating epigenetic phenomena into the models. For example, we show that, once reduction of Hsp90 activity induces a new phenotype, the new, potentially adaptive, phenotype can be inherited in the subsequent progeny for many generations, even without further reduction of Hsp90 activity [10]. In other words, only one bout of exposing variations in the parental generation is necessary, and the adaptive phenotype can be inherited in the progeny without further randomization by additional stress. Because they did not use a sensitized epigenetic system, Waddington [4,5], and Rutherford and Lindquist [3], needed several generations of stress before the new phenotypes were assimilated. Our model necessitates that a population undergoing selection must enter a bottleneck because only a small percentage of a stressed population would have an adaptive morphological variation. However, many models of evolution invoke that populations encounter a bottleneck before speciation can occur, such as the "African Eve" hypothesis for human evolution [53].

11. Future prospects

In a speculative methods paper, we have recently described quantitative epigenetic linkage (QEL) mapping experiments in an isogenic strain that we are pursuing to follow up our published epigenetics research [10,34]. We have also recently described how modern multi-generational epigenetic-mapping techniques can be used in the fields of cancer and obesity research [54]. The excitement of the resurgence of the field of epigenetics is summarized by Pigliucci, who said, "Nonetheless, it seems that genetic assimilation and epigenetics, after decades of neglect, are finally back on the center stage of evolutionary research. Perhaps they will remain in the spotlight long enough to be incorporated in mainstream evolutionary theory" [17].

Acknowledgements

We thank Colin Meiklejohn for helpful comments and suggestions and Jessica Treisman for pointing out that the ectopic X-Gal staining was in the hemocytes adhering to the discs and not in the peripodial membrane, as we originally reported. Research in our labs are supported by NIH Grants (R01 AA12276, R01 GM63225, R21 ES11751, and R01 CA105349) to D.M.R., NIH Grants (DK57301, DK60821), an American Cancer Society Grant (RPG-96-13504-DDC) to X.L., an NIH Training Grant (T32-CA09678) to V.E.S., and a UAB-CNRC Grant to M.D.G.

References

[1] Waddington CH. Canalization of development and the inheritance of acquired characters. Nature 1942;150:563–5.
[2] Meiklejohn CD, Hartl DL. A single mode of canalization. Trends Ecol Evol 2002;17:468–73.
[3] Rutherford SL, Lindquist S. Hsp90 as a capacitor for morphological evolution. Nature 1998;396:336–42.
[4] Waddington CH. Genetic assimilation of an acquired character. Evolution 1953;7:118–26.
[5] Waddington CH. Genetic assimilation of the bithorax complex. Evolution 1956;10:1–13.
[6] Rendel JM. Canalisation and gene control. New York: Elsevier Science and Technology Books; 1968.
[7] Rendel JM. Canalization of the scute phenotype in *Drosophila*. Evolution 1959;13:425–39.
[8] Gilbert SF. Developmental biology, 6th ed. Sunderland, MA: Sinauer Associates, Inc.; 2000.
[9] Queitsch C, Sangster TA, Lindquist S. Hsp90 as a capacitor of phenotypic variation. Nature 2002;417:618–24.
[10] Sollars V, et al. Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution. Nat Genet 2003;33:70–4.
[11] Cossins A. Cryptic clues revealed. Nature 1998;396:309–10.
[12] Stearns SC. Progress on canalization. Proc Natl Acad Sci USA 2002;99:10229–30.
[13] Mitchell-Olds T, Knight CA. Evolution. Chaperones as buffering agents? Science 2002;296:2348–9.
[14] Marshall E. Plant genetics. A hidden *Arabidopsis* emerges under stress. Science 2002;296:1218.
[15] Vel'kov VV. New insights into the molecular mechanisms of evolution: stress increases genetic diversity. Mol Biol (Mosk) 2002;36:277–85.
[16] Wagner GP, Chiu CH, Hansen TF. Is Hsp90 a regulator of evolvability? J Exp Zool 1999;285:116–8.
[17] Pigliucci M. Epigenetics is back! Hsp90 and phenotypic variation. Cell Cycle 2003;2:34–5.
[18] Rutherford SL, Henikoff S. Quantitative epigenetics. Nat Genet 2003;33:6–8.
[19] Sangster TA, Queitsch C, Lindquist S. Hsp90 and chromatin: where is the link? Cell Cycle 2003;2:166–8.
[20] Lamarck JBP. Zoological philosophy (1809). Chicago: University of Chicago Press; 1984.
[21] Cannon HG. Lamarck and modern genetics. Weisport, CT: Greenwood Publishing Group, Inc.; 1975.
[22] Darwin C. In: Suriano G, editor. The origin of the species (1959). New York: Random House; 1998.
[23] Mendel G. Experiments in plant hybridisation (1865). Boston: Harvard University Press; 1965.
[24] Allen GE. Thomas Hunt Morgan: a scientific biography. Princeton, NJ: Princeton University Press; 1978.
[25] Morgan TH. Mechanism of Mendelian heredity. New York: Johnson Reprint Corporation; 1915.
[26] Medvedev ZA. Rise and fall of T.D. Lysenko. New York: Columbia University Press; 1969.
[27] Sheldon CC, et al. The control of flowering by vernalization. Curr Opin Plant Biol 2000;3:418–22.
[28] Schlaroo W. Canalization: genetic and developmental aspects. Annu Rev Ecol Systemat 1991;22:65–93.
[29] Bateman KG. The genetic assimilation of the dumpy phenocopy. J Genet 1959;56:341–51.
[30] Yahara I. The role of HSP90 in evolution. Genes Cells 1999;4:375–9.
[31] Freeman BC, Yamamoto KR. Disassembly of transcriptional regulatory complexes by molecular chaperones. Science 2002;296:2232–5.

[32] Schotta G, et al. Position-effect variegation and the genetic dissection of chromatin regulation in *Drosophila*. Semin Cell Dev Biol 2003;14:67–75.
[33] Bateman KG. The genetic assimilation of four venation phenocopies. J Genet 1957;56:443–7.
[34] Garfinkel MD, Sollars VE, Lu X, Ruden DM. Towards a quantitative epigenetics. Meth Mol Biol 2003, in press.
[35] Hirsch J. Studies in experimental behavior genetics: II. Individual differences in geotaxis as a function of chromosome variations in synthesized *Drosophila* populations. J Comp Physiol Psychol 1959;52:304–8.
[36] Toma DP, White KP, Hirsch J, Greenspan RJ. Identification of genes involved in *Drosophila* melanogaster geotaxis, a complex behavioral trait. Nat Genet 2002;31:349–53.
[37] Carvalho AB, et al. Y chromosome and other heterochromatic sequences of the *Drosophila* melanogaster genome: how far can we go? Genetica 2003;117:227–37.
[38] Peifer M, Polakis P. Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science 2000;287:1606–9.
[39] Gibson MC, Schubiger G. Peripodial cells regulate proliferation and patterning of *Drosophila* imaginal discs. Cell 2000;103:343–50.
[40] Gibson MC, Schubiger G. *Drosophila* peripodial cells, more than meets the eye? Bioessays 2001;23:691–7.
[41] Hirsch HV, Mercer J, Samaziotis H, Huber M, Stark DT, Torno-Morley T, et al. Behavioral effects of chronic exposure to low levels of lead in *Drosophila* melanogaster. Neurotoxicology 2003;24:435–42.
[42] Hirsch HV, et al. Developmental visual plasticity in *Drosophila*. Ann NY Acad Sci 1991;627:359–62.
[43] Morley EJ, et al. Effects of chronic lead exposure on the neuromuscular junction in *Drosophila* larvae. Neurotoxicology 2003;24:35–41.
[44] Erkine AM, Gross DS. Dynamic chromatin alterations triggered by natural and synthetic activation domains. J Biol Chem 2003;278:7755–64.
[45] Maresca B, Lindquist S. Heat shock. New York: Springer-Verlag; 1991. p. 320.
[46] Foster PL. Adaptive mutation: implications for evolution. Bioessays 2000;22:1067–74.
[47] Feder ME, Hoffmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 1999;61:243–82.
[48] Weiss K. Goings on in Mendel's garden. Evol Anthropol 2002;11: 40–4.
[49] Wilkinson GS, Presgraves DC, Crymes L. Male eye span in stalk-eyed flies indicates genetic quality by meiotic drive suppression. Nature 1998;391:276–8.
[50] Grimaldi D, Fenster G. Evolution of extreme sexual dimorphisms: structural and behavioral convergence among broad-headed male Drosophilidae (Diptera). Am Museam Novitates 1989;2939: 1–25.
[51] Gould SJ. The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptionist programme. Proc R Soc Lond B: Biol Sci 1979;205:581–98.
[52] Gould SJ. The exaptive excellence of spandrels as a term and prototype. Proc Natl Acad Sci USA 1997;94:10750–5.
[53] Lewin R. The unmasking of mitochondrial eve. Science 1987;238: 24–6.
[54] Garfinkel MD, Ruden DM. Chromatin effects in nutrition, cancer, and obesity. J Nutr 2003, in press.
[55] Gibson M, Lehman D, Schubiger G. Lumenal transmission of decapentaplegic in *Drosophila* imaginal discs. Dev Cell 2002;3: 451.