Evidence for an epigenetic mechanism by which Hsp90 acts as a capacitor for morphological evolution

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Morphological alterations have been shown to occur in *Drosophila melanogaster* when function of Hsp90 (heat shock 90-kDa protein 1α, encoded by *Hsp83*) is compromised during development[1]. Genetic selection maintains the altered phenotypes in subsequent generations[1]. Recent experiments have shown, however, that phenotypic variation still occurs in nearly isogenic recombinant inbred strains of *Arabidopsis thaliana*[2]. Using a sensitized isogenic *D. melanogaster* strain, *iso-Kr*$^{If-1}$, we confirm this finding and present evidence supporting an epigenetic mechanism for Hsp90's capacitor function, whereby reduced activity of Hsp90 induces a heritably altered chromatin state. The altered chromatin state is evidenced by ectopic expression of the morphogen wingless in eye imaginal discs and a corresponding abnormal eye phenotype, both of which are epigenetically heritable in subsequent generations, even when function of Hsp90 is restored. Mutations in nine different genes of the *trithorax* group that encode chromatin-remodeling proteins also induce the abnormal phenotype. These findings suggest that Hsp90 acts as a capacitor for morphological evolution through epigenetic and genetic mechanisms.

The *D. melanogaster* segmentation gene *Kruppel* (*Kr*) encodes a zinc-finger transcription factor that is required for the development of several thoracic and abdominal segments[3]. The dominant *Kr*$^{Irregular\ facets}$ (*Kr*$^{If-1}$) allele causes ectopic expression of Kr protein mostly in the ventral region of the eye imaginal disc, which causes a reduction in the size of the eye (refs. 4,5; Fig. 1*a*,*b*). In a deficiency (Df) chromosome screen, the details of which will be presented elsewhere, we found that deficiency for ten chromosomal regions caused ectopic outgrowth in eyes of *Kr*$^{If-1}$ flies. When we mated females with any of these deficiencies (Df/+) to *Kr*$^{If-1}$ homozygous males, a considerable percentage of the *Kr*$^{If-1/+}$ offspring had ectopic outgrowth (data not shown). But the reciprocal cross (*Kr*$^{If-1}$ homozygous females × Df/+ males) produced at least an order of magnitude fewer offspring with ectopic outgrowth, suggesting that the phenotype is induced primarily by dosage-sensitive maternal-effect loci. Consistent with this genetic designation, ectopic outgrowth was independent of inheritance of the Df chromosome. Thorough testing of previously identified mutations uncovered by these Df lines identified 15 mutations in ten maternal-effect genes, dubbed *Me(Kr*$^{If-1}$*)*, that cause ectopic outgrowth. Although we tested mutations in multiple genes that were uncovered by the deficiencies, we identified only one *Me(Kr*$^{If-1}$*)* gene in each Df region (Table 1).

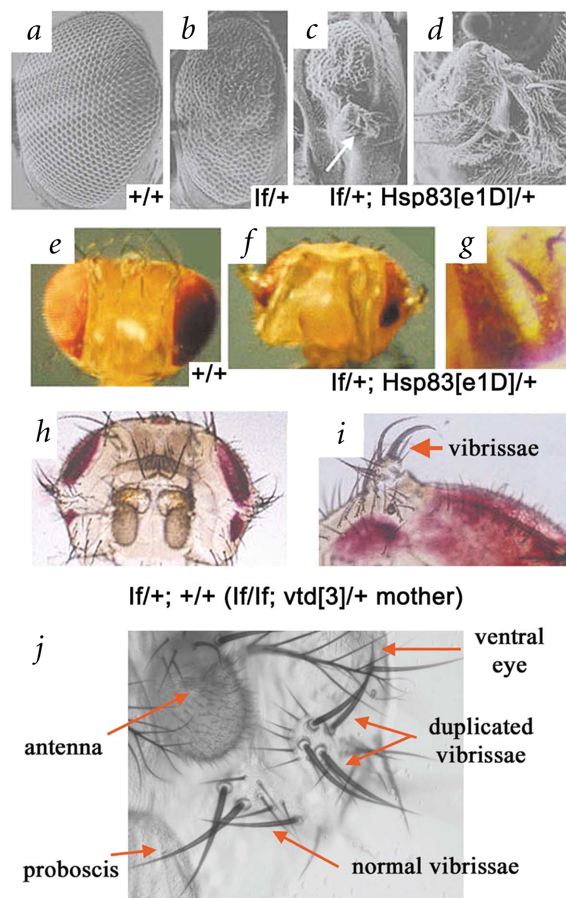

Fig. 1 Maternal loss of one copy of *Hsp83* can generate ectopic outgrowth in *D. melanogaster* eyes. ***a***,***b***, Scanning electron micrographs of eyes from a wild-type fly (*a*) and a *Kr*$^{If-1/+}$ fly (*b*) showed that the *Kr*$^{If-1/+}$ eye was smaller and rougher. ***c***, Scanning electron micrograph of a typical eye from a *Kr*$^{If-1/+}$; *Hsp83*$^{e1D/+}$ (If/+; Hsp83[e1D]/+) fly from parents with the genotypes *Hsp83*^{e1D}/*TM6B,Sb* (mother) and *Kr*$^{If-1}$/*Kr*$^{If-1}$ (father) showed ectopic outgrowth (arrow; enlarged in ***d***). ***e***,***f***, Light micrographs of heads from a wild-type (**+/+**) fly (e) and a fly with ectopic outgrowth as in *c* (*f*, high magnification in ***g***). ***h***, Cuticle preparation of a head of a fly with ectopic outgrowth mounted in Hoyers[22] and squeezed under a coverslip for 48 h at 65 °C. The fly had the genotype *Kr*$^{If-1/+}$; *TM6B,Sb*/+ (If/+; +/+) from parents with the genotypes *Kr*$^{If-1}$/*Kr*$^{If-1}$; *vtd*^{3}/*TM6B,Sb* (mother) and Oregon R (father). ***i***, Enlarged view of a head of a fly with ectopic outgrowth, prepared as in *h*. ***j***, Light micrograph of the ventral region of an eye with a mild ectopic outgrowth, prepared as in *h*. The normal vibrissae, the ectopic vibrissae, an antenna, a proboscis and the ventral region of an eye are indicated (arrows).

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Table 1 • *Me(Kr$^{\text{If-1}}$)* mutations identified in a genetic screen

Me(Kr$^{\text{If-1}}$)	Ectopic outgrowth*	Protein product
brahma^{2} (*brm^{2}*)	5 ± 1%	Chromatin binding
devenir^{2} (*dev^{2}/btl^{2}*)	10 ± 3%	FGF receptor
fs(1)h1^{1}	10 ± 2%	Chromatin binding
hsp83^{e3A}	11 ± 3%	Chaperone
hsp83^{e4A}	6 ± 2%	Chaperone
hsp83^{e6A}	6 ± 2%	Chaperone
hsp83^{e6D}	13 ± 4%	Chaperone
hsp83^{e1D}	15 ± 4%	Chaperone
khotalo^{1} (*kto^{1}*)	15 ± 4%	Chromatin binding
kismet^{1} (*kis^{1}*)	<0.1%	Chromatin binding
osa^{2}	10 ± 2%	Chromatin binding
skuld^{2} (*skd^{2}*)	2 ± 1%	Unknown
Trithorax-like^{85} (*Trl^{85}*)	10 ± 2%	Chromatin binding
urdur^{2} (*urd^{2}*)	5 ± 2%	Unknown
verthandi^{3} (*vtd^{3}*)	55 ±8%	Unknown
zeste^{V778} (*z^{V778}*)	5 ± 2%	Chromatin binding
iso-1,2,3	<0.1%	Isogenic strain
iso-Kr$^{\text{If-1}}$	<0.1%	Isogenic strain with *Kr$^{\text{If-1}}$*

*Percentages are the mean ± s.d. for ten replicate vials at 25 °C crossing mutation/balancer virgin female × *iso-Kr$^{\text{If-1}}$* male. In all cases, the number of F1 progeny scored was >500. The strains *kis^{1}*, *iso-1,2,3* and *iso-Kr$^{\text{If-1}}$* did not show any transformation phenotype in over 500 F1 progeny. As *kis* is a TrxG gene, this indicates that not all mutations in TrxG genes induce ectopic outgrowth. All strains have mutations in TrxG genes except those with the *hsp83* alleles and the last two.

For all of the *Me(Kr$^{\text{If-1}}$)* mutations, the most severe ectopic-outgrowth phenotype that we observed was crude appendage-like structures protruding from the ventral region of the eye (Fig. 1*e*–*g*). The ectopic outgrowth is probably not an appendage, however, because vibrissae-like bristles were also apparent on most outgrowths (Fig. 1*h*,*i*). The least severe ectopic-outgrowth phenotype consisted of a duplication of vibrissae bristles that are normally present ventral to the antennae. The vibrissae consist of two large sensory bristles and an adjacent row of smaller bristles (Fig. 1*j*). We scored a fly as having ectopic outgrowth if at least one of the eyes had at least four large ectopic bristles on it.

Most of the *Me(Kr$^{\text{If-1}}$)* mutations that we identified were in *trithorax* group (TrxG) genes[6] (Table 1). TrxG genes affect both epigenetic inheritance of acetylated chromatin and global gene-expression patterns[7]. TrxG proteins are thought to be involved in the maintenance of 'active' highly acetylated chromatin and act in a manner opposite to Polycomb group (PcG) proteins that are involved in the maintenance of 'inactive' under-acetylated chromatin[7]. Notably, in addition to TrxG mutations, we found that five alleles of *Hsp83*, encoding a chaperone in the Hsp90 family[8], had *Me(Kr$^{\text{If-1}}$)* activity (Table 1 and Fig. 1*c*,*d*,*f*,*g*). The involvement of a chaperone protein in the chromatin regulatory machinery is not unprecedented, as heat shock cognate 4 (Hsc4) was recently identified as part of the PcG multi-protein complex and *hsc*-mutant flies had a *Pc*-like phenotype[9].

The TrxG mutation *verthandi^{3}* (*vtd^{3}*) caused the highest frequency of ectopic outgrowth (Table 1). In a *vtd^{3}* selection experiment (Fig. 2*a*), we investigated whether ectopic outgrowth generated by the *vtd^{3}* mutation was selectable in the same manner as are phenotypes induced by reduced levels of Hsp90 (ref. 1). We determined whether the *vtd^{3}* mutation was required to establish as well as maintain ectopic outgrowth. If the *vtd^{3}* mutation was not required for maintenance, then the outgrowth would persist even in *vtd^{+}/vtd^{+}* progeny. Our results indicate that *vtd^{3}* was not required for maintenance; upon selection, an increasing percentage of *vtd^{+}/vtd^{+}* flies had ectopic outgrowth (Fig. 2*b* and Web Table A online). In this experiment we could not determine whether selection was mediated by an epigenetic or a genetic mechanism because the *Kr$^{\text{If-1}}$*; *vtd^{3}* and *iso-1,2,3* P0 strains had different genetic backgrounds (Fig. 2). We attempted to correct this shortcoming in the next series of experiments.

To test the proposed requirement of genetic variation for generating abnormal phenotypes, we made an isogenized *Kr$^{\text{If-1}}$* strain, *iso-Kr$^{\text{If-1}}$*, that has very little genetic variation and used geldanamycin, a potent and specific inhibitor of Hsp90 (ref. 1), to induce ectopic outgrowth. In this geldanamycin selection experiment (Fig. 2*a*), we fed *iso-Kr$^{\text{If-1}}$* males and females either normal medium or medium containing 3.56 μM geldanamycin. Geldanamycin was required for generating ectopic outgrowth (see Web Table A online). We then selected individual F1 *iso-Kr$^{\text{If 1}}$* males and virgin females with ectopic outgrowth and bred them together for 13 generations in the absence of the drug. As previously observed with outbred strains of *D. melanogaster*[1], we found that the percentage of flies with ectopic outgrowth was higher in each successive generation and reached a plateau by the F6 generation (Fig. 2*b*). This plateau was maintained until the experiment was terminated at the F13 generation (Fig. 2*b* and Web Table B online). We conclude that, in contrast with previous propositions[1], genetic variation is probably not required for selection and 'fixation' of ectopic outgrowth.

Next, we identified *wingless* (*wg*) as a probable 'target gene' of *Me(Kr$^{\text{If-1}}$)* mutations and the peripodial membrane as a probable 'target tissue'. We analyzed Wg expression because this morphogen is required for many developmental processes ranging from embryonic segmentation to limb development[10]. The peripodial membrane is a squamous-epithelial layer that covers the eye disc. Other morphogens, such as the TGFβ-family member Decapentaplegic (Dpp) and Hedgehog (Hh), have been shown to signal from the peripodial membrane to the columnal layer that forms the eye[11]. To analyze Wg expression, we used the enhancer-trap strain *wg^{02657}-lacZ*, which expresses β-galactosidase in a pattern that is

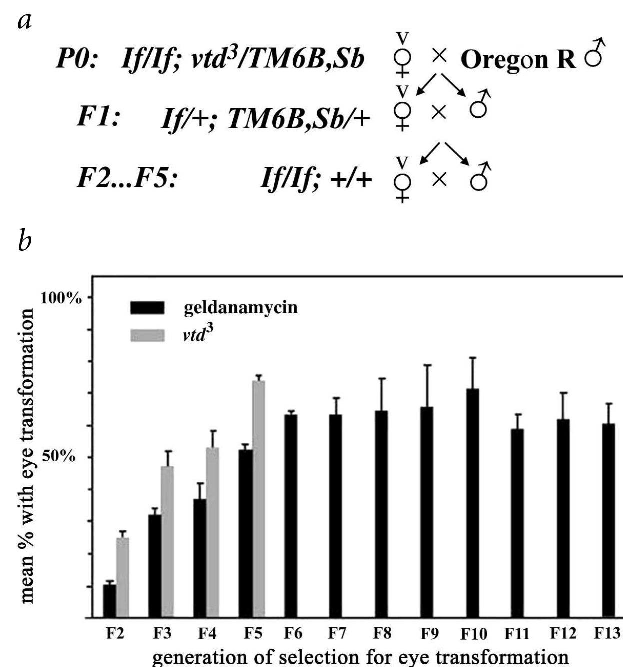

Fig. 2 *vtd^{3}* and geldanamycin selection experiments showed higher frequency of outgrowth in later generations. ***a***, Genetic crosses for the *vtd^{3}* selection experiment. The *vtd^{3}* mutation was present only in the P0 generation. ***b***, Selection increased the percentage of flies with ectopic outgrowth in both the *vtd^{3}* selection experiment (gray bars) and the geldanamycin selection experiment (black bars). Error bars indicate the s.e. for the progeny of replicate matings. The number of flies scored in each cross is shown in Web Tables A and B online.

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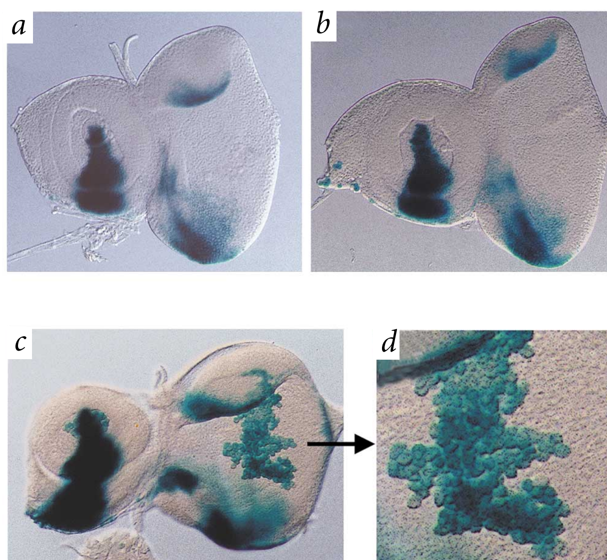

Fig. 3 Ectopic expression of *wg*^{02657}-*lacZ* was induced by *Hsp83* mutations. ***a*–*c***, Eye-antennal imaginal discs stained with X-gal from a third-instar larva (iso/wg-Z) with a *iso-1,2,3* mother and a *wg*^{02657}-*lacZ/CyO* father (*a*), from a third-instar larva (wg-Z/e1D) from a *wg*^{02657}-*lacZ/CyO* mother and a *Hsp83*^{e1D}/*TM6B,Sb* father (*b*) and from a third-instar larva (e1D/wg-Z) with a *wg*^{02657}-*lacZ/CyO* father and an *Hsp83*^{e1D}/*TM6B,Sb* mother (*c*). Ectopic X-gal staining in the large squamous epithelial cells in the peripodial membrane that covers the columnar epithelial cells of the eye disc (indicated by box; enlargement shown in ***d***) occurred in roughly 20% (14 of 72) of the third-instar larvae from the latter cross. The antennal disc is on the left and the eye disc on the right.

indistinguishable from endogenous Wg expression[12]. As expected, we observed normal Wg expression in more than 99% of eye discs from control flies (Fig. 3*a*) and from *wg*^{02657}-*lacZ* mothers and *Hsp83*-mutant fathers (Fig. 3*b*). In roughly 20% of third-instar larvae from *Hsp83*-mutant mothers, we observed ectopic *wg*^{02657}-*lacZ* expression in the peripodial membrane (Fig. 3*c*,*d*) in addition to the normal Wg pattern. This level of ectopic *wg*^{02657}-*lacZ* expression is consistent with the roughly 15% occurrence of ectopic outgrowth that we observed with *Hsp83*^{e1D} in a similar genetic cross (Table 1), suggesting that ectopic Wg contributes to ectopic outgrowth in eyes of *Kr*$^{If-1}$ flies.

Because the TrxG mutation *vtd*^{3} was also identified as an *Me(Kr*$^{If-1}$*)* mutation (Table 1), we investigated whether the ectopic *wg*^{02657}-*lacZ* expression was induced by *vtd*^{3}. We observed ectopic *wg*^{02657}-*lacZ* expression in over 50% of the third-instar larval eye discs derived from *Kr*$^{If-1}$; *vtd*^{3}/*TM6B,Sb* mothers and *wg*^{02657}-*lacZ* fathers, but in only about 1% of eye discs from the reciprocal cross (data not shown). Consistent with this observation, roughly 55% of the progeny from *Kr*$^{If-1}$; *vtd*^{3}/*TM6B,Sb* mothers and *wg*^{02657}-*lacZ* fathers had ectopic outgrowth (Table 1), whereas only about 1% of the progeny from the reciprocal cross did. Experiments investigating a possible molecular mechanism by which ectopic Wg expression causes ectopic outgrowth will be presented elsewhere (V.S. and D.M.R., manuscript in preparation).

Because TrxG mutations are thought to cause hypoacetylation of histones and *vtd* is an *Me(Kr*$^{If-1}$*)* gene, we investigated whether histone deacetylase (HDAC) inhibitors suppressed ectopic outgrowth. First, we showed that white pupae that had been treated with 4.5 µM of the potent HDAC inhibitor trichostatin A as larvae had roughly 50% more histone H3 acetylation compared with untreated pupae (Fig. 4*a*,*b*). It has previously been shown that flies fed a high concentration of the HDAC inhibitor sodium butyrate have more acetylated histones[13,14].

Next, we fed the HDAC inhibitors trichostatin A and sodium butyrate to F6 flies from the geldanamycin selection experiment (after the ectopic outgrowth reached the plateau level) to determine whether ectopic outgrowth would be suppressed. As expected, in the absence of HDAC inhibitors, roughly 65% of the progeny had ectopic outgrowth (Fig. 4*c*). In contrast, in the presence of 4.5 µM trichostatin A, only about 25% of the progeny had ectopic outgrowth (Fig. 4*c*), and in the presence of 10 mM sodium butyrate, which is effective only at much higher concentrations than trichostatin A, about 35% of the progeny had ectopic outgrowth (Fig. 4*c*). We observed a similar suppression of the ectopic *wg*^{02657}-*lacZ* phenotype at these concentrations of trichostatin A and sodium butyrate (Fig. 4*d*). Trichostatin A and sodium butyrate also suppressed ectopic outgrowth and ectopic *wg*^{02657}-*lacZ* expression induced by the *Me(Kr*$^{If-1}$*)* mutations *Hsp83*^{e1D} and *vtd*^{3} (data not shown).

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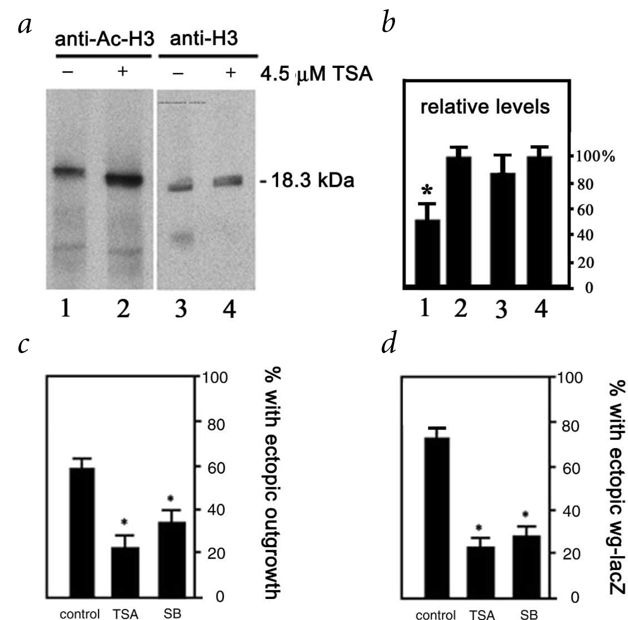

Fig. 4 HDAC inhibitors lowered the frequency of ectopic outgrowth in flies. ***a***, Western blot of protein from two white pupae from control Oregon R flies and from flies with 4.5 µM trichostatin A (TSA) in their food (+). The left image shows a filter probed with antibody against acetylated histone H3 (anti-Ac-H3), and the right image shows the same filter stripped and re-probed with antibody against histone H3 (anti-H3). ***b***, Quantification of triplicate western blots done as in *a*. There was roughly 50% more acetylated histone H3 present in the pupae raised on food containing trichostatin A than in the control. Numbers 1–4 indicate lanes marked in *a*. Asterisks indicate significant differences ($P < 0.05$, *t*-test) relative to the control. The total amount of histone H3 was not significantly affected by trichostatin A treatment ($P = 0.2$ relative to control, *t*-test). ***c***, Individual F7 virgin females with ectopic outgrowth from the geldanamycin selection experiment were mated to wild-type *iso-1,2,3* males in five vials. No drug (control), 4.5 µM trichostatin A (TSA) or 10 mM sodium butyrate (SB) was added to their food. The mean ± s.e. of the percentage of progeny with ectopic outgrowth are shown. Asterisks indicate significant differences ($P < 0.05$, *t*-test) relative to the control. ***d***, HDAC inhibitors trichostatin A and sodium butyrate reduced the frequency of ectopic *wg*^{02657}-*lacZ* expression in eye discs. Individual F7 virgin females from the geldanamycin selection experiment were mated to *wg*^{02657}-*lacZ/CyO* males in five vials. No drug (control), 4.5 µM trichostatin A (TSA) or 10 mM sodium butyrate (SB) was added to their food. The mean ± s.d. of the percentage of third-instar larvae with ectopic *wg*^{02657}-*lacZ* expression in eye discs are shown. Asterisks indicate significant differences ($P < 0.05$, *t*-test) relative to the control. One-tenth the dose of either trichostatin A or sodium butyrate had no significant effect on the frequency of ectopic outgrowth or the ectopic *wg*^{02657}-*lacZ* phenotype. The effects of higher doses of either trichostatin A or sodium butyrate could not be determined because twice the dose of either drug caused early white-pupal lethality in more than 90% of the progeny (data not shown).

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Is the genetic variation present in the *iso-Kr*$^{If-1}$ strain sufficient to explain the results of the geldanamycin selection experiment? We do not believe so, because a similarly isogenized strain used for sequencing *D. melanogaster* genomic DNA was found to have very little genetic variation[15]. According to data from Celera Genomics, an upper limit for the amount of single-nucleotide polymorphisms (SNPs) in the sequenced strain was estimated to be one SNP per 21 kb (A. Clark, pers. comm.). We conclude that genetic variation probably cannot account for the phenotypic selection that we observed.

In light of our data, we propose a refinement of the 1942 evolutionary 'canalization' model of Waddington[16] to an 'epigenetic canalization' model. In the canalization model[16], environmental stress induces a novel phenotype, and selection of existing genetic variation in subsequent generations allows fixation of the novel phenotype. According to Waddington, "[b]y such a series of steps, then, it is possible that an adaptive response can be fixed without waiting for the occurrence of a mutation which, in the original genetic background, mimics the response well enough to enjoy a selective advantage"[16]. In our epigenetic canalization model, we propose that an environmental stress causes a reduction in Hsp90 levels and, through some unknown interaction with TrxG proteins, induces an immediate 'chromatin effect'. Our model allows an adaptive response to be 'fixed' epigenetically, and therefore obviates the need to wait for the selection of existing genetic variation. In other words, it predicts a more rapid evolutionary process than is required for selection of existing genetic variation.

Because of the inherent instability of epigenetic inheritance, fixation of an epigenetically-determined phenotype is probably less stable than fixation through a genetic selection mechanism. Waddington, for example, was unable to reduce the frequency of the crossveinless phenotype in negative selection experiments once the phenotype was fixed[17]. In contrast, after only two or three generations of negative selection, we observed a complete reversion to wild-type frequency of ectopic outgrowth in our sensitized *iso-Kr*$^{If-1}$ strain in the geldanamycin selection experiment (data not shown). Similarly, epigenetic traits such as color variegation or cold adaptation in plants are unstably inherited[18,19]. Therefore, a combination of both epigenetic and genetic mechanisms is probably required to explain the rapid changes in body plans that are observed in the fossil record[20].

Methods

***D. melanogaster* stocks.** Strains with the independently isolated ethylmethane sulfonate–induced *Hsp83* alleles (*Hsp83*^{e3A}, *Hsp83*^{e4A}, *Hsp83*^{e6A}, *Hsp83*^{e6D} and *Hsp83*^{e1D}) were from T. Laverty[8]. The b^1 *Kr*$^{If-1}$ strain, the *wg*^{02657}*-lacZ/CyO* strain, the multiple marker strain b^1 dac^1 pr^1 cn^1 wx^{wxt} bw^1, the *iso-1,2,3* strain w^{1118}*; iso-2*; *iso-3* and the wild-type strain (Oregon R) were from the Bloomington Stock Center. We generated the *iso-Kr*$^{If-1}$ strain by mating b^1 dac^1 pr^1 cn^1 wx^{wxt} bw^1 *Kr*$^{If-1}$ /*iso-2* virgin females to b^1 dac^1 pr^1 cn^1 wx^{wxt} bw^1 males. We isolated F1 males with the genotype *iso-2* *Kr*$^{If-1}$/ b^1 dac^1 pr^1 cn^1 wx^{wxt} bw^1 that had all of the second chromosome between *b* (34D1) and *bw* (59E2) from the *iso-2* chromosome and part of the remaining chromosome (59E2-60F5) from the *Kr*$^{If-1}$ chromosome (*Kr*$^{If-1}$ is at 60F3). We then mated these males to *iso-1,2,3* virgin females to generate a stock with the genotype *iso-1; iso-2*, *Kr*$^{If-1}$/*iso-2*. The third chromosome was made isogenic by crossing males from this stock to females with the genotype *iso-1*; *iso-2*; *iso-3* /*TM6B,Sb* to generate flies with the genotype *iso-1*; *iso-2, Kr*$^{If-1}$/*iso-2*; +/*TM6B,Sb*, and then mating males from this cross to *iso-1,2,3* females to generate iso-1; *iso-2*, *Kr*$^{If-1}$; *iso-3* /*TM6B,Sb* flies. We then mated together males and virgin females from the last cross and selected progeny with the genotype *iso-1*; *iso-2*, *Kr*$^{If-1}$/*iso-2*; *iso-3* to keep as the *iso-Kr*$^{If-1}$ progenitor stock. Finally, we backcrossed virgin females from the *iso-Kr*$^{If-1}$ progenitor stock to *iso-1,2,3* males and repeated this for ten generations to generate the *iso-Kr*$^{If-1}$ strain, which has most of the DNA (>99%) from the *iso-1,2,3* strain except for the region immediately around *Kr*$^{If-1}$.

Genetic screen for *Me(Kr*$^{If-1}$*)*. The genetic screen for *Me(Kr*$^{If-1}$*)* mutations will be described elsewhere (V.S. and D.M.R., manuscript in preparation). To quantify ectopic outgrowth, we classified a phenotype as positive if there were at least four large ectopic bristles protruding from the ventral region of the eye or negative if there were three or fewer large ectopic bristles. As *Kr*$^{If-1/+}$ flies rarely have a single large bristle protruding from the ventral region of the eye (about 1 in 500 eyes), this classification strategy accurately identified a modified phenotype.

The *Me(Kr*$^{If-1}$*)* mutations differ from the zygotic *Su(Kr*$^{If-1}$*)* and *E(Kr*$^{If-1}$*)* mutations described previously, which cause an increase or a decrease, respectively, in the number of ommatidia in *Kr*$^{If-1}$/+ eyes[4,5], but do not cause ectopic outgrowth. A second difference is that the *Me(Kr*$^{If-1}$*)* mutations cause ectopic outgrowth regardless of whether they are inherited in the progeny (data not shown), whereas *E(Kr*$^{If-1}$*)* and *Su(Kr*$^{If-1}$*)* mutations must be present in the affected progeny[4,5]. A third difference is that *Me(Kr*$^{If-1}$*)* mutations primarily cause ectopic outgrowth when the mutation is present in the mother, whereas *E(Kr*$^{If-1}$*)* and *Su(Kr*$^{If-1}$*)* have identical effects when they are inherited from either parent[4,5].

Histology and pharmacology. We carried out light and scanning electron microscopy as previously described[21]. We dissected eye-antennal imaginal discs from third-instar larvae and stained them with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma) as previously described[22]. We dissolved geldanamycin and trichostatin A (Sigma) in 50% ethanol immediately before use and added it to 10 ml of standard cornmeal-agar food[22] in 30-ml vials (Applied Scientific). We stored sodium butyrate (Sigma) as a 1 M stock solution. We used antibodies against histone H3 and against acetylated histone H3 (Cell Signaling Technology) according to the manufacturer's specifications. In geldanamycin selection experiments, we fed 3.56 μM geldanamycin to P0 flies with the genotype *iso-Kr*$^{If-1}$. In the selection process, we mated F1 virgin females with ectopic outgrowths in individual vials without drug to F1 males with ectopic outgrowths (F1 × F1), and repeated this in triplicate until the F13 generation was scored for ectopic outgrowth.

Statistical analyses. For each of ten sublines designated A–J in the *vtd*^{3} selection experiment, we determined the percentage of males and females in generations F2–F5 that had ectopic outgrowth (see Web Table A online). We then used this percentage as the dependent variable and generation number as the independent variable in one-way ANOVA and all available post-hoc tests using SPSS 10. The ANOVA *F*-test statistic was 30.02 ($P \approx 0.000$, d.f. = 3 between groups, d.f. = 36 within groups), indicating that the variance between groups was greater than the variance within groups. The Levene statistic indicated non-homogeneous variances (Levene statistic = 4.907, d.f.1 = 3, d.f.2 = 36). Multiple comparisons using the most liberal of the post-hoc tests (least significant difference (LSD), essentially all pairwise *t*-tests) indicated that the baseline F2 generation was highly significantly different from all three subsequent generations, a conclusion that was supported by more conservative post-hoc analyses such as Bonferroni, Tukey–HSD and Scheffe. The post-hoc tests also divided the *vtd*^{3} selection generations into three homogeneous subsets, one comprised of generation F2, another of F3 and F4 and the last of F5. This neatly coincided with visual and intuitive assessments of the four generations.

Treatment of *iso-Kr*$^{If-1}$ flies with geldanamycin produced three male and three female flies with ectopic outgrowth (see Web Table B online), constituting 1.34% of the F1 generation. We used these flies to establish three single-pair sublines designated A, B and C. We then selected offspring with ectopic outgrowth from each subsequent generation until the F13 progeny were recovered. For each of the three sublines, we determined the percentage of negative *iso-Kr*$^{If-1}$ males, negative *iso-Kr*$^{If-1}$ females, positive males with ectopic outgrowth and positive females with ectopic outgrowth in generations F2–F13 (see Web Table B online). As in the *vtd*^{3} selection experiment, we used this percentage as the dependent variable and generation number as the independent variable in one-way ANOVA and all available post-hoc tests. Visual and intuitive assessments suggest that selection increased the proportion of flies with ectopic outgrowth from a baseline value of 10.43 ± 1.04% in generation F2 to a plateau of roughly 65% in generations F6–F13. Generations F3–F5 showed intermediate proportions of flies with ectopic outgrowth. This interpretation is supported by the statistical analyses. The ANOVA *F*-test statistic, which compares variance between groups to variance

within groups, was 7.35 ($P \approx 0.000$, d.f. = 11 between groups, d.f. = 24 within groups), but the Levene statistic was 2.973 (d.f.1 = 11, d.f.2 = 24), indicating that the variances were not homogeneous. This complicates interpretation of most post-hoc tests. Taking the F2 generation as baseline, the most liberal of the post-hoc tests (LSD) indicated that F2 significantly differed from F3 ($P = 0.032$), that F2 highly significantly differed from all others ($P \leq 0.011$; see Web Table B online) and that generations F6–F13 did not significantly differ from each other ($0.2 < P < 0.999$). More conservative post-hoc analyses (Bonferroni, Tukey–HSD and Scheffe) corroborated these conclusions. Post-hoc tests also divided the 12 generations into homogenous subsets. The first subset comprised generations F6–F13 (in which we observed the plateau of ectopic outgrowth), and the second subset comprised generation F2. The third group, generations F3–F5 (in which we observed intermediate percentages of ectopic outgrowth), could be grouped with either of the other subsets, implying that they were indeterminate.

Note: Supplementary information is available on the Nature Genetics website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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