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Epigenetically Heritable Alteration of Fly Development in Response to Toxic Challenge

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SUMMARY

Developing organisms have evolved a wide range of mechanisms for coping with recurrent environmental challenges. How they cope with rare or unforeseen challenges is, however, unclear as are the implications to their unchallenged offspring. Here, we investigate these questions by confronting the development of the fly, *D. melanogaster*, with artificial tissue distributions of toxic stress that are not expected to occur during fly development. We show that under a wide range of toxic scenarios, this challenge can lead to modified development that may coincide with increased tolerance to an otherwise lethal condition. Part of this response was mediated by suppression of Polycomb group genes, which in turn leads to derepression of developmental regulators and their expression in new domains. Importantly, some of the developmental alterations were epigenetically inherited by subsequent generations of unchallenged offspring. These results show that the environment can induce alternative patterns of development that are stable across multiple generations.

INTRODUCTION

Although the ability of the environment to modify the patterns of development is well recognized, environmental influences are usually studied in the context of stimuli that have occurred repeatedly during evolution (e.g., changes in temperature, food supply, interactions with predators, etc.). These inputs invoke specific regulatory modules that have been selected during evolution to mount beneficial solutions to changes in environmental conditions (Abouheif and Wray, 2002; D'Orazio et al., 2006; Dodson, 1989; Emlen and Nijhout, 2001; Nijhout, 1991; Woodward and Murray, 1993). However, in addition to frequently occurring environmental stimuli, organisms may sometimes encounter rare or even unforeseen challenges. In these cases the organism may not have an efficient solution, and the challenge could potentially lead to detrimental effects. How developing organisms cope with environments that are very rare or unforeseen is, however, poorly understood.

Coping with stressful conditions during the process of development may lead to deviations from the regular process. Such deviations are normally suppressed by mechanisms of canalization or robustness, the buffering of phenotypes against environmental and genetic perturbations (Waddington, 1942, 1957; Rutherford and Lindquist, 1998; Gilbert and Epel, 2008; West-Eberhard, 2003). Although this buffering is crucial for maintaining adapted phenotypes, it may compromise the ability of organisms to accommodate challenging new environments (Wagner, 2005). How this dichotomy between plasticity and robustness is resolved is a fundamental but nonetheless poorly explored aspect of development.

Developmental changes that are induced by the environment have been traditionally thought to be nonheritable. However, recent evidence of epigenetic inheritance phenomena in a variety of species (Jablonka and Raz, 2009; Jablonka and Lamb, 1995; Rando and Verstrepen, 2007) suggests that the environment might be able to induce epigenetic variations that are stable across multiple generations. Yet, the scope and mechanisms of transgenerational epigenetic inheritance of changes that are induced by the environment are not clear, especially with respect to rare or previously unforeseen environments.

Studying the influence of unforeseen environments on multiple generations of the organism could be achieved by creating artificial setups of environmental challenge. These setups could shed light on the interplay between robustness and emergence of altered phenotypes, and may offer a powerful paradigm for investigating forces that modify developmental patterns on the timescales of few generations (as opposed to comparisons of diverging organisms that focus on evolutionary time scales; Alonso and Wilkins, 2005; Carroll, 2008; Davidson and Erwin, 2006; Prud'homme et al., 2006; Shubin et al., 2009). Indeed, recent work with a microorganism (the yeast, *S. cerevisiae*) demonstrated remarkable ability of the cells to physiologically modify their regulatory network so as to mount adaptive heritable responses to a novel challenge (Stolovicki et al., 2006; Stern et al., 2007).

Here, we introduce an experimental model for investigating how a developing multicellular organism, the fly *D. melanogaster*, responds to artificial tissue distributions of toxic stress. Using this model, we show that the regulation of development is flexible enough to deviate from the normal course of development in a way that may confer tolerance to the otherwise lethal challenge. At the same time, the epigenetic change in regulation can lead to modified developmental outcomes. We

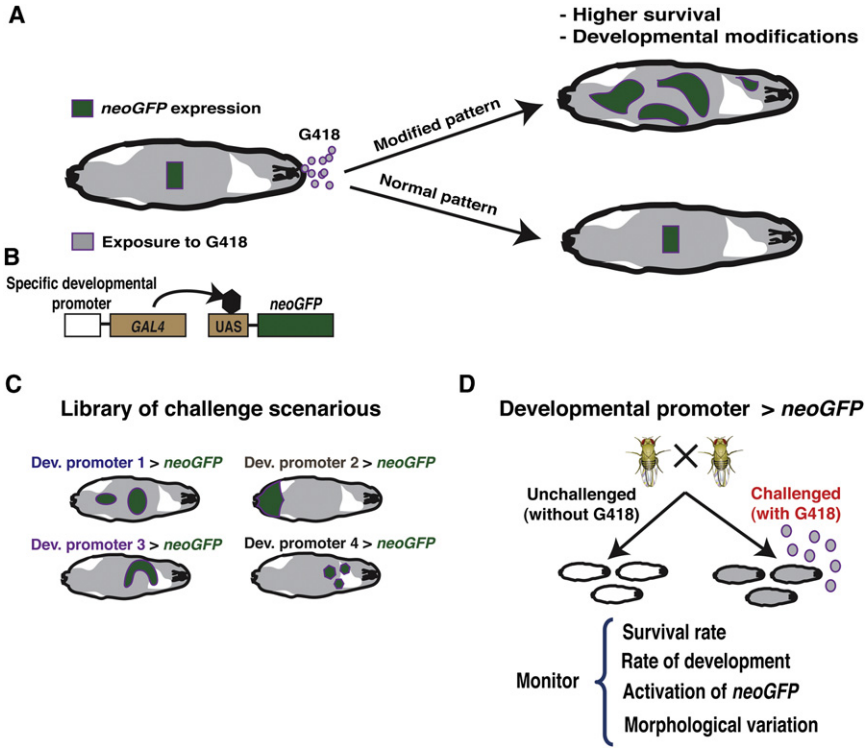

Figure 1. Presentation of a Toxic Challenge to Developing Fly Larvae

(A) Left view shows a scheme for creating artificial distribution of toxic stress across the larvae. Right view illustrates potential scenarios that include extension of the endogenous domain of activation of the developmental promoter (top), and no change (bottom).

(B) *neoGFP* is expressed using the GAL4-UAS system.

(C) Different distributions of toxic stress are generated by applying G418 to a library of fly lines, each expressing the resistance gene (*neoGFP*) under the regulation of a different promoter.

(D) A scheme of the basic experiment.

further show that this response is enabled by reduction in Polycomb group (PcG) genes, which in turn leads to derepression of developmental regulators and their activation in new domains. Strikingly, some of the modified developmental phenotypes were epigenetically inherited by subsequent generations of offspring that did not experience the challenge.

RESULTS

Confronting Fly Development with Artificial Patterns of Toxic Challenge

We developed a drug-antidrug model for confronting the development of fly larvae with artificial distributions of toxic stress across the larva. This was implemented by supplementing the food with G418 at concentrations that are lethal to wild-type larvae and placing a resistance gene fused to *GFP* (*neoGFP*), under the regulation of an arbitrary, spatiotemporally restricted developmental promoter (Figure 1). In this setup, a toxic stress is experienced in tissues that are exposed to G418 but do not express sufficient levels of *neoGFP*. Because the promoters controlling the resistance gene are arbitrarily chosen, the distribution of toxic stress in the larva is unnatural. This setup creates a severe challenge to the developing larvae because the activation patterns of the arbitrarily chosen developmental promoters are not normally related to G418 and are not activated in all the exposed tissues. Without extension in activation of the developmental promoters into new domains, the larvae may not develop into viable adults (Figure 1A, right).

Because the response to this challenge may include a general reaction to G418 and a more specific response that depends on the choice of promoter controlling the resistance gene, we investigated different choices of promoters. For that, we crossed UAS-*neoGFP* flies to existing fly lines expressing *GAL4* under the regulation of specific promoters (Figure 1B). Exposure to G418 in these promoter-*GAL4::UAS-neoGFP* larvae leads to a promoter-specific distribution of toxic stress (Figure 1C). For these fly lines we analyzed the survival rate, the duration of larval development, the patterns of expression of *neoGFP*, and the morphology of the flies (Figure 1D).

Survival analysis under G418 concentration that is lethal to wild-type larvae revealed a substantial ability to withstand the challenge: in eight out of nine different cases of restricted promoters driving the expression of *neoGFP*, the survival was significantly higher than in wild-type (Figure 2A). As expected, larvae with only the *UAS-neoGFP* or *GAL4* transgenes did not survive (Figure 2A, inset). On the other hand, *Act5C::neoGFP* larvae that express *neoGFP* in every single tissue were completely resistant to the challenge (Figure 2A, inset).

In one of the lines in which *neoGFP* was controlled by the *hairy* promoter, 100% of the larvae developed into adulthood, and the number of adult flies exposed to G418 was the same as without G418 (Figure 2A), indicating accommodation of the challenge without selection of individuals. Despite the remarkable G418 tolerance in this case, all the challenged larvae were delayed by ~2–3 days in their development compared with unchallenged larvae (Figure 2B; $p < 10^{-5}$).

Modification of Promoter Activations in Response to the Challenge

Because all the *hairy::neoGFP* larvae survived the G418 treatment and gave rise to the same number of adults as unchallenged larvae, we used the recruitment of *neoGFP* to *hairy* as a test case for studying physiological (selection-independent) effects of the challenge. First, we tested if the challenge can modify the domains of expression of *neoGFP* and the *hairy* gene in challenged versus unchallenged *hairy::neoGFP* (third-instar) larvae. We found that exposure to G418 elevated the

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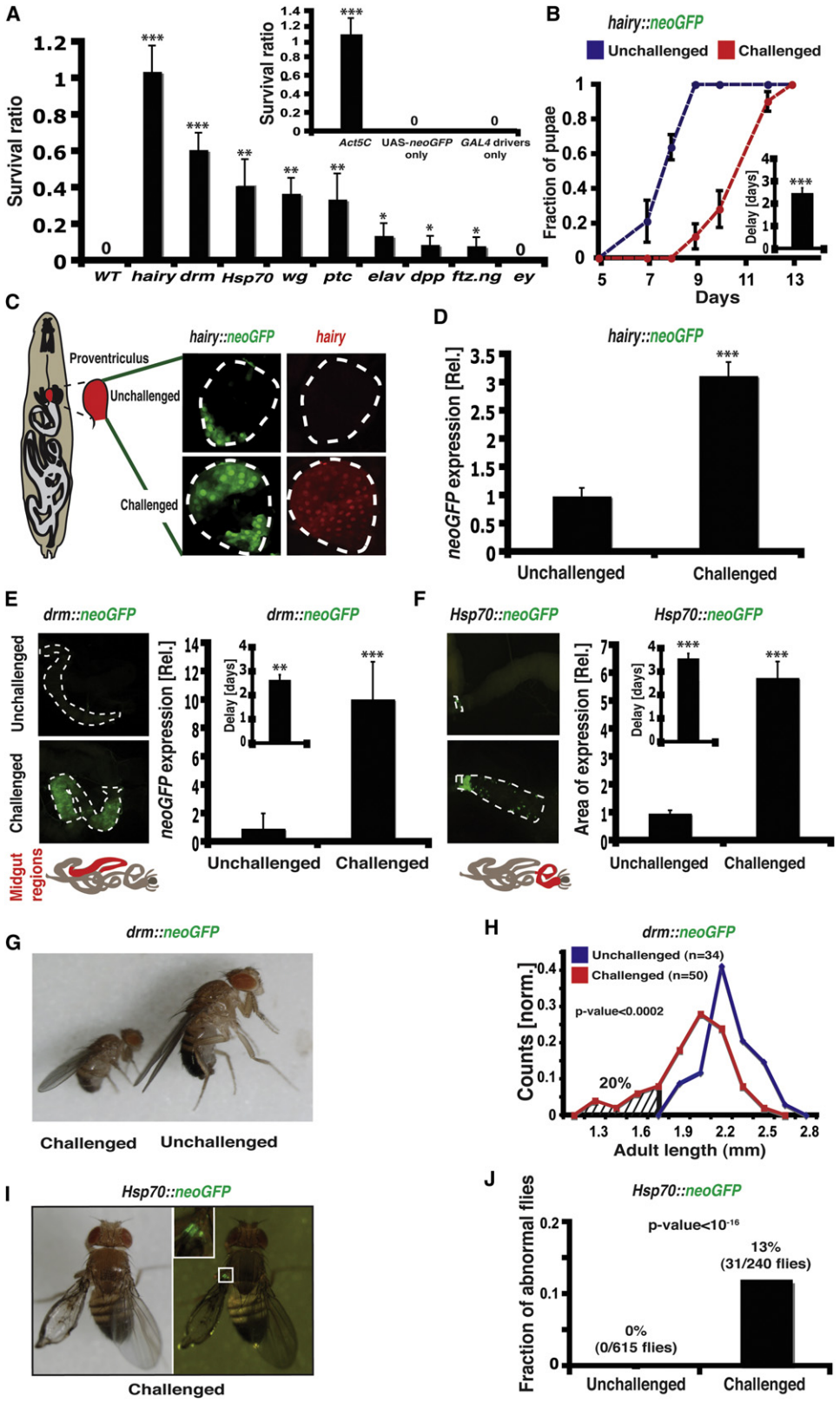

expression of *neoGFP*, and expanded it into new domains in the proventriculus (Figures 2C and 2D; $p < 10^{-5}$) and the midgut (Figure S1A). The significant shift of the *GFP* intensity histogram toward higher levels in challenged versus unchallenged larvae (Figure S1B) strongly suggests that the induction occurred in most or all the larvae of the first generation. Immunostaining the gut with antibodies against *hairy* showed that the endogenous expression of *hairy* was also elevated in the proventriculus of the challenged larvae, although the pattern of *hairy* induction was not identical to that of the *neoGFP* (Figure 2C). High-resolution mapping of the *hairy-GAL4* locus in the enhancer trap line (Brand and Perrimon, 1993) showed that the *GAL4* gene was inserted between the *hairy* gene and its upstream 14 kb regulatory element (Figure S1C). It is possible that this upstream regulatory element can activate the *GAL4* and *hairy* genes separately or together depending on tissue specific *trans*-acting factors. Thus, the noncomplete overlap of induction of the *neoGFP* and *hairy* might reflect tissue-specific activation by the upstream regulatory element.

To evaluate the generality of the broadening of promoter activation in response to the challenge, we analyzed the expression of *neoGFP* under eight different promoters. In five of these cases, the expression of *neoGFP* was elevated in promoter-specific regions of the gut (Figures 2C–2F and S1D), suggesting that the plastic response in the *hairy::neoGFP* system was not a special case but rather part of a widespread activation response. For example, placement of *neoGFP* under the control of the *drumstick* (*drm*) promoter led to induction of *neoGFP* in the midgut of challenged *drm::neoGFP* larvae, reflecting a change in regulation of the *drm* promoter (Figure 2E; $p < 10^{-5}$). Thus, a sizable group of developmental regulators that are usually regarded as firmly restricted to specific domains were actually found to be expressed in wider domains following the challenge. This extension of promoter activation was not restricted to developmental promoters and was also observed in the midgut of *Hsp70::neoGFP* larvae (Figure 2F; $p < 10^{-5}$). As in the *hairy::neoGFP* case, the induction of *neoGFP* in response to the challenge was associated with a significant delay in development in all these promoter-*GAL4* cases (insets in Figures 2E and 2F, and S1E).

We hypothesized that the broadenings of promoter activation in response to the challenge were independent of whether they led to a beneficial response. We verified this hypothesis for the *hairy* case by analyzing the expression of *hairy* in *Hsp70::neoGFP* larvae, which do not exhibit *neoGFP* induction in the proventriculus. Indeed, in this case the challenge induced the *hairy* protein in the proventriculus without a corresponding induction of *neoGFP* (Figure S1F). This showed that the induction of *hairy* was not specific to the *hairy::neoGFP* scenario and was independent of whether or not it led to a beneficial response.

To evaluate the contribution of *neoGFP* expression in the gut to the ability to survive the challenge, we analyzed the expression in a total of 13 *GAL4* lines (including lines with unspecified promoters but well-described expression patterns). Examination of expression patterns and survival showed that expression in the salivary glands, fat body, brain, and discs could confer little or no survival (Figures S2A–S2E). On the other hand, substantial expression in either the midgut or foregut (with or without expression in the other tissues) correlated with significantly higher survival ratio (Figures S2F–S2J).

Challenge-Induced Modification of the Adult Morphology

Analysis of adult morphology revealed that the modified activation of the developmental promoters did not always lead to changes in the adult form; for example, *hairy::neoGFP* flies that were challenged during their larval stages did not exhibit any obvious morphological defects, indicating that the adult morphology can be buffered against the change in *hairy* expression. This buffering allows modifications to occur at the gene expression level without necessarily impacting higher levels of functionality. Still, this buffering is not without limits; in some of

Figure 2. Challenge-Induced Modifications in Development

(A) Promoter-specific survival of fly lines. Survival ratio represents the number of adults developed with versus without G418. Data are represented as mean survival ratio ±SE measured in N_v vials pooled from N_e replicated experiments. N_v and N_e for each line are as follows: *hairy* ($N_v = 33$, $N_e = 9$); *drm* (24, 7); *Hsp70* (9, 4); *wg* (9, 5); *ptc* (4, 2); *elav* (5, 3); *dpp* (7, 3); *ftz* (7, 3); *WT* (*yw*, 15, 4). Inset shows larvae carrying only the GAL4 driver (pool of six *GAL4* lines, $N_v = 22$, $N_e = 7$), only the UAS-*neoGFP* transgene ($N_v = 15$, $N_e = 4$), and both (case of *Act5C::neoGFP*; $N_v = 11$, $N_e = 4$).

(B) Representative delay of 2–3 days in the development of challenged compared to unchallenged *hairy::neoGFP* larvae. Data are represented as mean fraction of pupae ±SE in six vials. Inset shows quantification of the mean delay ±SE in six replicated experiments.

(C) Representative images of induced expression of *neoGFP* (green) and the endogenous *hairy* protein (red) in the proventriculus of challenged third-instar *hairy::neoGFP* larva.

(D) Quantification of *neoGFP* expression (mean GFP intensity ±SE) in the proventriculi of challenged (n = 23) versus unchallenged (n = 31) *hairy::neoGFP* larvae pooled from six replicated experiments.

(E) Representative images (left) and quantification of *neoGFP* expression (right) in the midgut of challenged (n = 15) versus unchallenged (n = 23) *drm::neoGFP* larvae pooled from three replicated experiments. Inset shows quantification of developmental delay of challenged versus unchallenged larvae in seven replicated experiments.

(F) Same as (E) for challenged (n = 13) versus unchallenged (n = 18) *hsp70::neoGFP* larvae. Data are represented as mean area of expression ±SE. Inset shows quantification of developmental delay in five replicated experiments.

(G) Example of a dwarf *drm::neoGFP* adult fly that was exposed during development to 400 μg/ml of G418 (left) compared to a nonexposed fly (right).

(H) Histogram of adult lengths for challenged versus unchallenged *drm::neoGFP* flies.

(I) Representative image of challenged *Hsp70::neoGFP* fly with one abnormal wing and a corresponding induction of *neoGFP* expression at the base of the wing (inset).

(J) Incidence of wing abnormalities in challenged (200–400 μg/ml of G418) and unchallenged *Hsp70::neoGFP* adult flies pooled from seven experiments.

*$p < 0.05$, **$p < 0.001$, ***$p < 10^{-5}$ (Student's t test).

See Figures S1 and S2 as well.

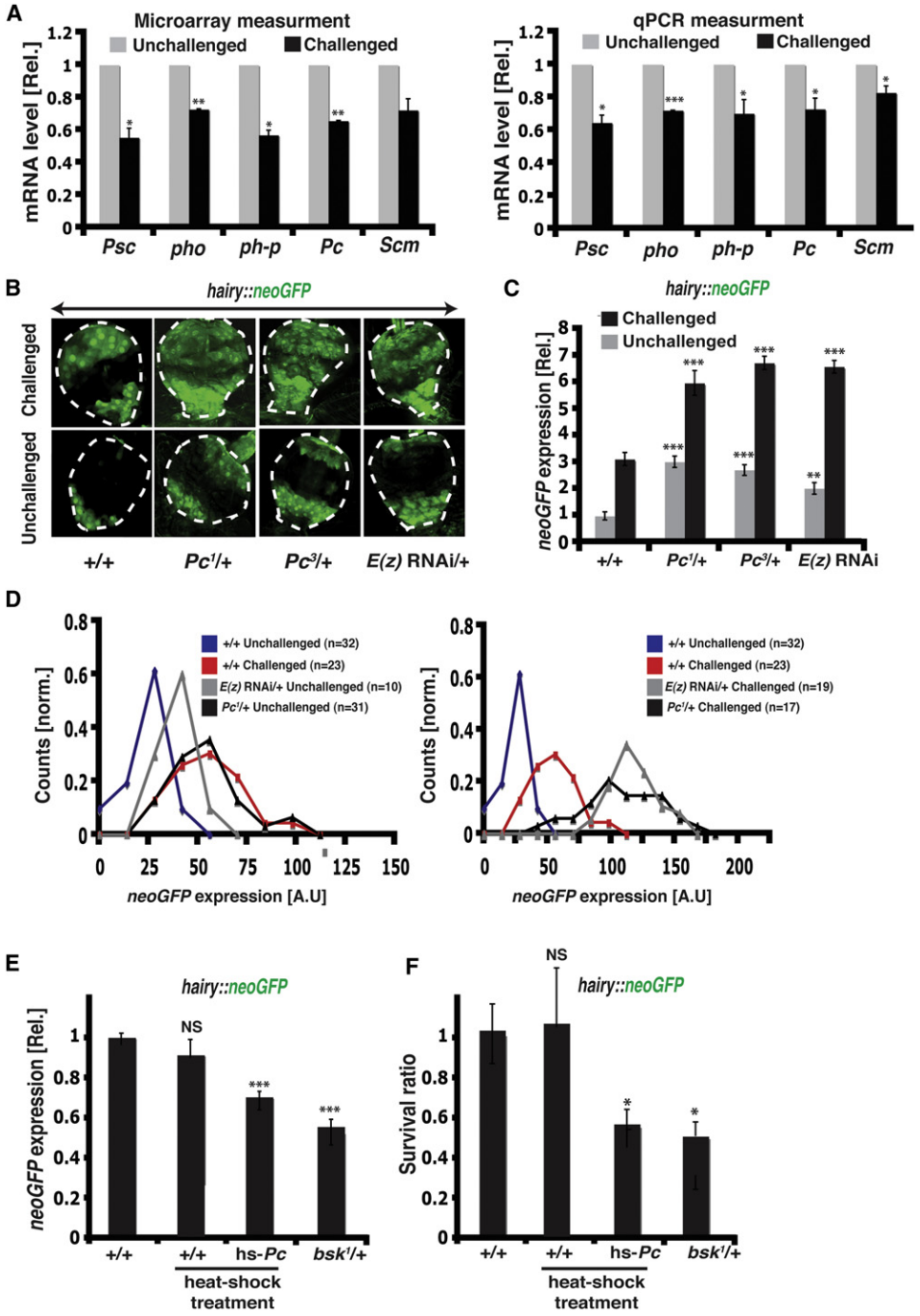

Figure 3. Modifications in the Regulation of *hairy* Are Mediated by Suppression of PcG Genes

(A) mRNA levels (mean ± SE) of PcG genes in the proventriculus of challenged versus unchallenged *hairy::neoGFP* third-instar larvae. mRNA was measured by Affymetrix microarrays (left, n = 2) and by qPCR (right, n = 3).

(B) Representative patterns of *neoGFP* expression in the proventriculus of challenged (top) and unchallenged (bottom) *hairy::neoGFP* larvae heterozygous for a mutation in *Pc* (Pc^1, Pc^3) or, alternatively, carrying a UAS-RNAi against *E(z)* (*E(z)i*/+).

(C) Statistics of the patterns of *neoGFP* expression (mean expression per proventriculus ±SE) based on the following numbers of proventriculi (n) pooled from at least three replicated experiments in each case (Unchallenged: +/+ n = 32, Pc^1/+ n = 32, Pc^3/+ n = 15, *E(z)i*/+ n = 10; Challenged: +/+ n = 23, Pc^1/+ n = 17, Pc^3/+ n = 25, *E(z)i*/+ n = 19).

(D) Left panel is normalized histograms of *neoGFP* expression in proventriculi of unchallenged and challenged nonmutant *hairy::neoGFP* larvae (blue and red, respectively), Pc^1 larvae (black), and larvae carrying *E(z)* RNAi (gray). Right panel is the same as the left panel with challenged Pc^1 (black), *E(z)* RNAi larvae (gray).

the other cases that we tested, the challenge did lead to gross morphological alterations. When *neoGFP* was placed under the regulation of the *drm* promoter, the challenged larvae developed into considerably smaller adult flies compared to unchallenged larvae (Figures 2G and 2H; $p < 2 \times 10^{-4}$), and the length of ~20% of the challenged flies was below the minimal length observed without the challenge (Figure 2H). An example of a very small (dwarf) fly that was developed from challenged *drm::neoGFP* larva is shown in Figure 2G (left). In a different scenario of challenge (case of *Hsp70::neoGFP*), ~13% of the challenged larvae developed into flies with wing abnormalities (Figures 2I and 2J; $p < 10^{-16}$). These deformations varied in severity and appeared on either one or both wings. In either case they were accompanied by a strong induction of *neoGFP* expression at the base of the abnormal wing, but never in a normal wing (Figure 2I, inset). Lack of morphological abnormalities in *Act5C::neoGFP* flies expressing *neoGFP* ectopically in every single tissue (0 of 459 flies, Figures S1G and S1H) showed that the morphological alterations in response to the challenge do not reflect any toxic effect of the *neoGFP* protein itself.

Notably, the changes in adult size and wing patterns were observed, respectively, only in the *drm::neoGFP* and *Hsp70::neoGFP* lines, and not in lines in which *neoGFP* is controlled by other promoters. Thus, the same agent of toxicity (G418) can induce different phenotypes that depend on the promoter controlling the resistance gene and, hence, are not determined only by the exposure to G418.

Modifications in the Regulation of *hairy* Are Mediated by Suppression of PcG Genes

The clear preference for broadening of the domain of promoter activation (five out of eight tested cases) as opposed to a decrease in activation (none of the cases) suggested a global derepression mechanism. This led us to test the involvement of the PcG genes, which are known to repress many developmental regulators by maintaining epigenetically repressed chromatin state across cell divisions (Ringrose and Paro, 2004; Schuettengruber and Cavalli, 2009). Using the recruitment of *neoGFP* to the *hairy* promoter as a test case, we found that the challenge downregulated the mRNA levels of the PcG genes *Polycomb* (*Pc*), *Pleiohomeotic* (*pho*), *Posterior sex comb* (*Psc*), *Sex comb on midleg* (*Scm*), and *Polyhomeotic proximal* (*ph-p*) in the larval foregut (Figure 3A). To test the implications of reduction in Polycomb to the regulation of *hairy*, we analyzed the expression pattern of *neoGFP* in *hairy::neoGFP* larvae carrying heterozygous mutation of *Pc*, an essential component of the Polycomb repressive complex 1 (PRC1). We found that two *Pc* mutant alleles, Pc^1 and Pc^3, mimicked the stereotyped induction of *neoGFP* in the proventriculus without exposing the larvae to G418 (Figures 3B and 3C; $p < 10^{-5}$). Applying the challenge on the background of Pc^1 or Pc^3 further increased the levels of *neoGFP* beyond those observed for wild-type *Pc* (Figures 3B and 3C; $p < 10^{-5}$). Expression of *neoGFP* was also induced in the proventriculus of challenged and unchallenged *hairy::neoGFP* larvae carrying RNAi against *Enhancer of Zeste*, *E(z)*, under the regulation of a UAS promoter (Figures 3B and 3C; $p < 10^{-5}$ and $p < 10^{-3}$, respectively). *E(z)* is a component of the PRC2 complex responsible for specifying Polycomb target sites by trimethylating lysine 27 on histone H3, $H3K27me^3$. Increase in *neoGFP* by knockdown of *E(z)* in tissues expressing the resistance gene, therefore, provides additional support for the involvement of Polycomb reduction in the induction of *neoGFP*. In addition, the interindividual variability of *GFP* intensity in the proventriculus was larger in all the experimental scenarios that involved reduction in Polycomb function (either exposure to G418, or the use of larvae with mutant or RNAi background; Figure 3D). This increase in variability suggests that suppression of Polycomb function relieves the strict control over the activation of the *hairy* promoter, thus increasing the variability of expression.

To test whether *Polycomb* gain of function has the inverse effect of blocking the induction of *neoGFP* and, hence, compromising the survival under challenge, we generated *hairy::neoGFP* flies with an extra allele of *Pc* controlled by the heat-shock promoter (*hs-Pc*). Using this line, we found that heat shock-mediated overexpression of *Pc* reduced the levels of *neoGFP* expression at the base of the proventriculi of unchallenged, *hairy::neoGFP hs-Pc* larvae compared with unchallenged larvae lacking the *hs-Pc* transgene (Figure 3E, *hs-Pc* with heat shock versus (+/+); $p < 10^{-5}$). As expected, the survival of *hairy::neoGFP hs-Pc* larvae under challenge was significantly reduced compared with challenged *hairy::neoGFP* larvae lacking the *hs-Pc* transgene (Figure 3F, *hs-Pc* with heat shock versus +/+; $p < 0.05$). In addition we verified that the heat shock itself did not alter the expression of *neoGFP* or the survival under challenge (Figures 3E and 3F, +/+ with heat shock versus +/+).

Because the increase in expression of a single Polycomb gene (*Pc*) may not necessarily result in gain of Polycomb function, we sought additional support based on work showing that activation of the JNK pathway can suppress Polycomb function (Lee et al., 2005; Owusu-Ansah and Banerjee, 2009). We therefore tested if the disruption of JNK signaling would reduce the expression of the resistance gene and, hence, compromise survival under challenge. Indeed, we found that unchallenged *hairy::neoGFP* larvae that are heterozygous mutant for the JNK MAP kinase gene, *basket* (bsk^1), exhibited lower expression of *neoGFP* at the base of the proventriculus (Figure 3E, bsk^1/+ versus +/+; $p < 10^{-5}$). As expected, the survival of these larvae was significantly reduced under challenge compared with the nonmutant larvae (Figure 3F, bsk^1/+ versus +/+; $p < 0.05$).

(E) Expression of *neoGFP* in proventriculi of unchallenged *hairy::neoGFP* larvae exposed to heat shock with or without *hs-Pc* (*hs-Pc*, n = 27; +/+, n = 10), on the background of *basket* mutation without heat shock (bsk^1, n = 17), and in unchallenged nonmutant larvae (n = 23). Data are represented as mean expression ±SE based on the number of proventriculi (n) pooled from at least three replicated experiments in each case.

(F) Survival ratios for the cases in (E) under exposure to 400 μg/ml of G418 (*hs-Pc*, n = 12 vials; +/+ with heat shock, n = 10; *bsk1*/+, n = 9; +/+ without heat shock, n = 33). Data are represented as mean survival ratio ±SE in fly vials pooled from four to nine replicated experiments.

*$p < 0.05$, **$p < 10^{-3}$, ***$p < 10^{-5}$ (Student's t test). NS, not significant.

See Figure S3 as well.

Unlike the Polycomb system, the *HP1* and *Su(var)3-9* heterochromatin proteins are not required for mediating the response to the challenge. Overexpression of *Su(var)3-9* using a heat-shock-inducible promoter (*hs-Su(var)3-9*) did not reduce the survival of *hairy::neoGFP* larvae under challenge (Figure S3). Additionally, overexpression of *HP1* using *hs-HP1* did not change the levels of *neoGFP* expression in the proventriculus (Figure S3). Furthermore, fly larvae that are heterozygous mutant for *HP1* or for *Su(var)3-9* or that are carrying RNAi against *HP1* did not show altered expression levels of *neoGFP* in the proventriculus (Figure S3). Thus, the induction of *neoGFP* in response to the challenge was mediated by suppression of *PcG* genes without involvement of *HP1* and *Su(var)3-9*.

Downregulation of Specific PcG Genes Mimics the Phenotypes in *drm::neoGFP* Larvae and Increases the Survival under Challenge

To verify that reduction in various PcG genes can expand the domain of activation of other promoters used in our study, we examined the development of *drm::neoGFP* larvae on the background of PcG RNAi. We found that RNAi against the gene *ph-p* (Dura et al., 1985) under the regulation of the *drm* promoter, *drm::(ph-p)i*, reproduced all the challenge phenotypes in larvae that were not exposed to G418; the unchallenged *drm::neoGFP* flies with *ph-p* RNAi exhibited (1) elevated levels of *neoGFP* in the larval midgut (Figures 4A and 4B; $p < 10^{-3}$); (2) delayed larval development (Figure 4C; $p < 0.05$); and (3) formation of very small pupae (~35% of the population; Figure 4D; $p < 0.03$). Note, however, that the *(ph-p)i* phenotype was too strong, and the pupae failed to eclose even without the challenge. Thus, in this case the reduction in PcG levels could not assist the survival because of the detrimental effect of Polycomb reduction regardless of the challenge.

To test if the reduction in other PcG genes could increase the survival under challenge, we examined the development of *drm::neoGFP* larvae on the background of RNAi against *pho*, a PhoRC component whose phenotype is known to be milder compared with other genes involved in Polycomb function (Brown et al., 2003; Simon et al., 1992). Hence, in the case of *pho*, the positive effect of *neoGFP* induction might exceed the detrimental effect of *pho* RNAi. Indeed, *pho* RNAi under the regulation of *drm* elevated the expression of *neoGFP* in the foregut (Figures 4E and 4F; $p < 10^{-5}$) and increased the survival of challenged *drm::neoGFP* larvae from ~57% to ~87% (Figure 4G; $p < 0.05$).

Notably, all four of the developmental promoters that were activated by the challenge (*hairy*, *elav*, *drm*, and *ftz.ng*) are natively located within ''Polycomb regions'' (blue region in Figure S4; Filion et al., 2010; Sexton et al., 2012). These chromosomal domains are enriched with binding of Polycomb proteins and trimethylation of H3K27 (Schuettengruber et al., 2009). Two of the four lines, *hairy-GAL4* and *elav-GAL4*, are enhancer trap lines and thus correspond to the endogenous regulation. The remaining two lines, *drm-GAL4* and *ftz.ng-GAL4*, are not enhancer traps, but their promoter regions contain sequences whose endogenous counterparts are enriched with *polyhomeotic* (*Ph*) binding sites, often considered more specific indicators of Polycomb response elements (Schuettengruber et al., 2009; Figure S4).

Altogether, these results suggest that the challenge expands the domains of activation of developmental promoters by decreasing the levels of PcG genes, thus alleviating some of the repression of the developmental genes. This reduction may result in an increase in activation of the promoters depending on the expression of *trans*-acting, tissue-specific factors (Figure 4H). Activation in the gut could in turn lead to increased survival due to ectopic expression of *neoGFP* in this critical tissue.

Induced Developmental Patterns Are Epigenetically Inherited across Multiple Generations

Motivated by a variety of reported cases of epigenetic inheritance (reviewed in Jablonka and Raz, 2009; Rando and Verstrepen, 2007), we examined the possibility of transgenerational inheritance of the response to the challenge. To this end, we self-crossed *hairy::neoGFP* flies that were exposed to G418 during their own development and examined the development of their progeny grown for multiple generations in a G418-free environment (Figure 5A). In each generation we compared larvae whose F1 ancestors were exposed to G418 to larvae from the same generation without past exposure of ancestors. Remarkably, the offspring larvae across multiple generations retained the elevated levels of *neoGFP* and *hairy* expression in the proventriculus (Figures 5B and 5C), demonstrating transgenerational persistence of the induced state. Likewise, the unchallenged offspring of challenged ancestor flies were delayed in development, as if they were themselves exposed to the challenge (Figures 5D and 5E; $p < 0.05$). The inheritance occurred even after a single generation of ancestor exposure and was observed also in homozygous *hairy::neoGFP* larvae (Figures S5A and S5B), as well as in a different transgenic line of UAS-*neoGFP* (Figure S5C). The induced state of *neoGFP* and the delay in development persisted for multiple generations without G418 before reverting to the normal phenotype (Figures 5B–5E and 6). A representative case of reversion after four generations of inheritance is displayed in Figure 5F. The persistence of the environmentally induced response across multiple generations without the challenge and the eventual reversion to the wild-type phenotypes strongly suggest the involvement of an epigenetic inheritance mechanism.

To evaluate the generality of inheritance, we examined additional cases of *promoter::neoGFP* flies. We found that *drm::neoGFP* larvae exhibited heritable induction of *neoGFP* expression in the midgut (Figures 6A and 6B) and heritable delay in development (Figures 6C and 6D). Importantly, a small fraction (~6%) of F2–F7 *Hsp70::neoGFP* offspring of flies that were challenged in F1 exhibited inheritance of wing abnormalities (Figures 6E and 6F; $p < 10^{-9}$). Owing to this low penetrance, the wing abnormalities were observed in some of the generations after exposure to G418. However, they were never observed in flies from the same generations (0 out of 708) without a history of exposure to G418 in their ancestors. In addition to inheritance of wing abnormalities, unchallenged *Hsp70::neoGFP* offspring with exposure to G418 in their ancestors exhibited a delay in development (Figures 6G and 6H).

To test if the inheritance of the response involved modifications in the transgenes, we again used the *hairy::neoGFP* line

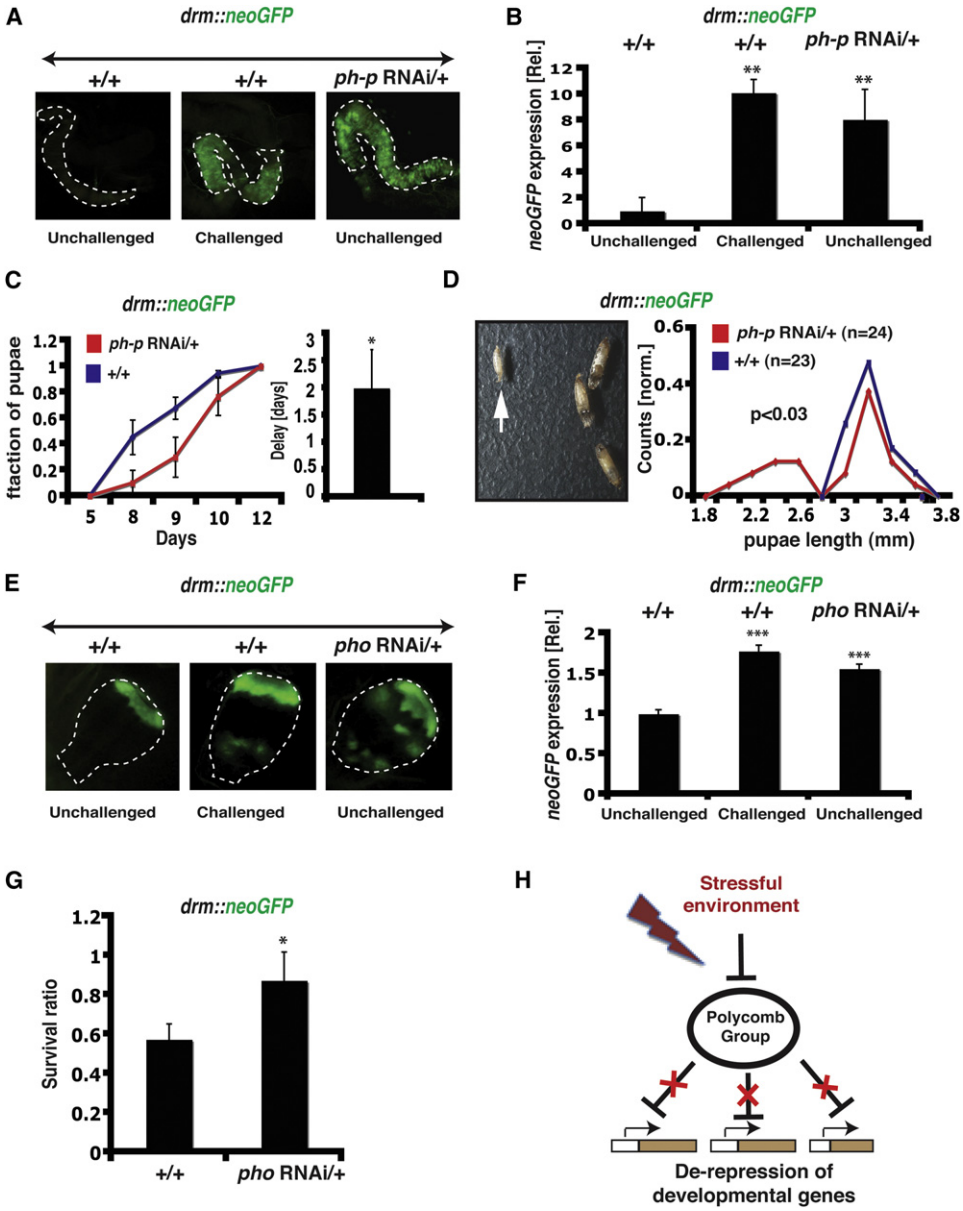

Figure 4. Downregulation of PcG Genes Can Mimic the Challenge Phenotypes in *drm::neoGFP* Flies and Increase the Survival under Challenge

(A) Representative images of *neoGFP* expression in the midgut of unchallenged *drm::neoGFP* larvae (left), challenged larvae (center, 400 μg/ml of G418), and *drm::neoGFP* larvae expressing RNAi against *ph-p* under the control of the *drm* promoter (right).

(B) Statistics of *neoGFP* expression for the cases described in (A). Data are represented as mean expression ±SE measured in midgut tissues pooled from three replicated experiments.

(C) Left view shows representative delay in development of unchallenged *drm::neoGFP* larvae with *ph-p* RNAi (compared with +/+). Right view illustrates mean delay ±SE in four replicated experiments.

(D) A significant fraction of unchallenged larvae carrying RNAi against *ph-p* form very small pupae. Left view is a representative image of a small pupa (white arrow). Right view shows normalized length histograms for *drm::neoGFP* pupae with and without *ph-p* RNAi pooled from three replicated experiments.

(E) Same as (A) for the proventriculus of larvae carrying RNAi against *pho* (right).

(F) Statistics of *neoGFP* expression for the cases described in (E). Data are represented as mean expression ±SE measured in proventriculi pooled from three replicated experiments.

(G) Survival of *drm::neoGFP* larvae with or without *pho* RNAi (*pho* RNAi, n = 10 vials; +/+, n = 24) Data are represented as mean survival ratio (versus unchallenged) ±SE measured in vials (n) pooled from three to six replicated experiments.

(H) Hypothesized model.

*$p < 0.05$, **$p < 0.001$, ***$p < 10^{-5}$ (Student's t test).

See Figure S4 as well.

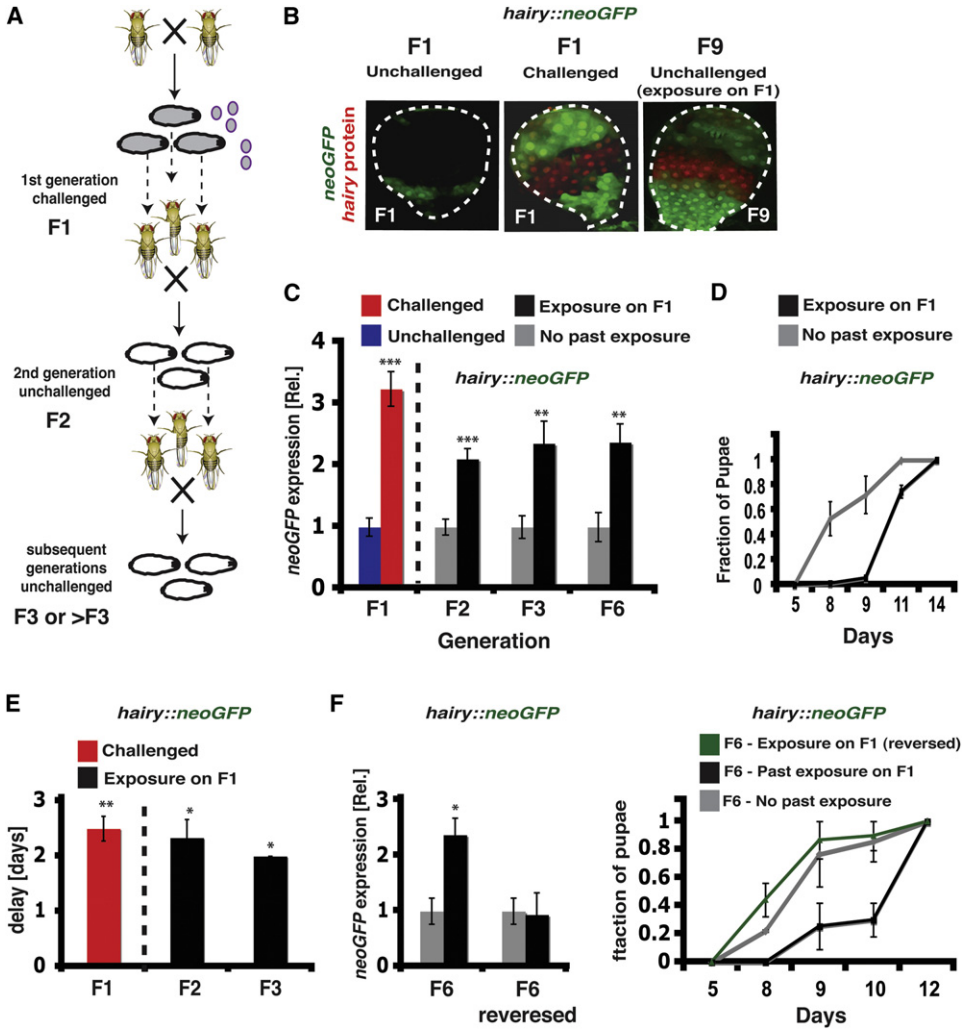

Figure 5. Induced Developmental Patterns Are Epigenetically Inherited across Multiple Generations

(A) A scheme of the basic inheritance experiment.

(B) Representative images demonstrating elevated expression of *neoGFP* (green) and *hairy* (red) in the proventriculus of challenged F1 *hairy::neoGFP* larva (center versus left), and in unchallenged F9 offspring following eight generations without G418 (right versus left).

(C) Statistics of *neoGFP* expression in the proventriculi of unchallenged (n = 31) and challenged (n = 23) F1 *hairy::neoGFP* larvae, and in unchallenged F2, F3, and F6 generations with (n = 61, 20, 22, respectively) or without history of exposure on the F1 generation (n = 50, 18, 20). Data are represented as mean expression per proventriculus ±SE based on the noted numbers of proventriculi (n) pooled from three to five replicated experiments. The dashed lines were drawn to separate results obtained for the F1 generation from those of later generations.

(D) Representative delay in development of unchallenged F10 *hairy::neoGFP* larvae with past exposure on F1 compared to larvae without past exposure.

(E) Statistics of the delay (mean ± SE in three to four replicated experiments). The dashed lines were drawn to separate results obtained for the F1 generation from those of later generations.

(F) Example of reversal of inheritance in F6 indicated by mean *neoGFP* expression in reversed (n = 22) and nonreversed (n = 22) F6 *hairy::neoGFP* larvae with past exposure on F1 (left). Reversal of the inheritance of the delay in development for the F6 *hairy::neoGFP* larvae (right).

*p < 0.05, **p < 10^{-3}, ***p < 10^{-5} (Student's t test).

See Figure S5 as well.

as a test case. We followed the development of unchallenged offspring of heterozygous *hairy-GAL4,* UAS-*neoGFP* flies that were exposed to G418. F6 offspring lacking both the *hairy-GAL4* and UAS-*neoGFP* transgenes were delayed in development as their *hairy::neoGFP* siblings (Figures S5D and S5E), indicating that the inheritance of the delay did not involve *cis* modifications at these loci. This conclusion was also supported by the inheritance of the delay in all the promoter-*GAL4* cases that were included in this study.

A priori, the persistence of the induced phenotypes does not necessarily require inheritance of the initial mediator of the response. We therefore tested if the suppression of Polycomb, which mediates the induction of *hairy*, is heritable in our system. Comparing the levels of PcG genes in the proventriculi of

unchallenged F3 *hairy::neoGFP* larvae with or without exposure to G418 in their F1 ancestors revealed that the suppression of PcG genes was nonheritable (Figure S5F). Nevertheless, the F3 larvae with exposure to G418 in their F1 ancestors did exhibit inheritance of the delay in development and *neoGFP* induction in the proventriculus (data not shown). This result confirmed that the outcome of a developmental change can be heritable without heritability of the presumed mediator (Polycomb suppression). It is plausible that the suppression of Polycomb in the challenged generation leads to chromatin modifications that persist across generations without the initial inducer.

The Response to the Challenge Is Composed of Heritable and Nonheritable Changes in Gene Expression

In addition to the effect of the challenge on developmental patterns, exposure to G418 may invoke detoxification responses and other types of classical responses to stress. To detect these responses and examine their heritability, we analyzed the genome-wide mRNA profiles in proventriculi of challenged versus unchallenged F1 larvae, as well as in unchallenged F3 offspring that inherited the induction of *neoGFP* and the delay in development. Comparison between proventriculi of challenged and unchallenged F1 larvae revealed a very strong detoxification response only in the challenged larvae. This response (that was insufficient to rescue the wild-type larvae) was manifested by over 10-fold induction of a battery of glutathione S-transferases (GSTs) (Figure 7A), a highly conserved set of genes known to reduce and thereby neutralize a wide range of toxins (Li et al., 2008; Low et al., 2007). However, unlike the heritable developmental phenotypes, the generic detoxification response was not heritable; the levels of all the GST genes in unchallenged F3 larva were similar with or without exposure to G418 in the F1 ancestors (Figure 7A). More comprehensive analysis of overlap between 266 genes that were induced over 2-fold in the challenged proventriculi, and 187 "general stress response" genes that were previously shown to be induced in at least 3 of 4 different stress conditions (Sørensen et al., 2005; Kristensen et al., 2005; Landis et al., 2004), revealed a relatively small but statistically significant overlap of 33 genes (Figure 7B; Table S1; $p < 10^{-11}$). This overlap confirmed the involvement of a classical stress response that coexisted with the modified developmental features. However, as with the GST genes, none of the 33 stress response genes was induced in a heritable manner (Figure 7C; $p < 10^{-22}$). The complete lack of heritability of the detoxification response showed that the inherited features do not reflect persistence of toxic stress across generations but rather responses that are epigenetically inherited by future generations of unstressed larvae. A similar difference in heritability was provided by the response to reduced temperatures during development; unchallenged, *hairy*::*neoGFP* larvae developed at 20°C exhibited a delay that was comparable to G418-challenged larvae grown at 25°C (Figure S6). However, the delay in response to reduced temperatures was not heritable and did not lead to induction of *neoGFP* (Figure S6), indicating that the induction of *hairy* in challenged larvae is not a simple by-product of delayed development.

Analysis of mRNA expression changes beyond detoxification and stress genes revealed an overall tendency for inheritance of expression changes in genes that responded to the challenge in F1. Indeed, genes that were either up- or downregulated in challenged (versus unchallenged) F1 tended to change in the same direction in the unchallenged F3 offspring of challenged F1 ancestors (R = 0.56; Figure 7D). The set of heritably modified genes included a clear signature of genes involved in the response to Ecdysone, including *Eip74EF*, *broad* (*br*), *Eip78C*, *ImpE3*, *E23*, *Hsp27*, *Hsp26*, and *Hsp67Bc* (Figure 7E, left). The levels of all these Ecdysone response genes were downregulated in challenged F1 and remained low in unchallenged F3. The heritable reduction in Ecdysone response genes was accompanied by heritable upregulation of aldehyde dehydrogenase, *Aldh*, which has been implicated in suppression of Ecdysone signaling (Halme et al., 2010), and of *CG31974*, a homolog of the *Bombyx mori* Eckinase (*Eck*) gene, which phosphorylates and thereby inactivates 20E Ecdysone (Schwedes et al., 2011) (Figure 7E, left). The heritable reduction in Ecdysone response genes and increase in Ecdysone suppressors are consistent with the heritable delay in development. Additional genes with heritable change span a variety of functions, including for example the neuropeptide and pheromone receptor (*Nplp2* and *Obp49a*), acetate and fatty acid metabolism (*AcCoAS* and *Lip3*), regulation of transcription (*slbo*), and chitin metabolism (*obst-A*) (Figure 7E, middle). Yet, the majority of the genes that exhibited heritable changes have unknown function (examples shown in Figure 7E, right).

Overall, these results show that the response to the challenge includes heritable and nonheritable components. Some of the phenotypes are common to all the scenarios of challenge (e.g., the delay in development), and yet others (such as dwarf flies and abnormal wings) depend on the choice of *promoter::neoGFP* setup.

DISCUSSION

Artificial Challenges as a Paradigm for Studying Deviations from Normal Development

Although developmental plasticity can be defined as the ability of the organism to modify its shape, state, movement, and rate of activity in response to environmental inputs (West-Eberhard, 2003), it is important to distinguish two classes of plastic responses. The first involves changes in response to stimuli that have been encountered repeatedly during evolution and for which the organism has an effective response program. The second type involves responses to rare or unfamiliar environments, for which the organism may not have an effective program. These responses might appear when the environment changes in an unfamiliar, stressful manner, or following a genetic change that compromises the normal progression of development and results in a novel scenario. A practical approach for modeling such scenarios is to engineer defying conditions that are unexpected to occur during normal development. Here, we introduced and used an experimental paradigm in which we expose fly larvae to artificially determined patterns of effective stress. The model involves different settings in which a drug (G418) generates toxic stress in arbitrarily defined (promoter-specific) tissues that do not normally

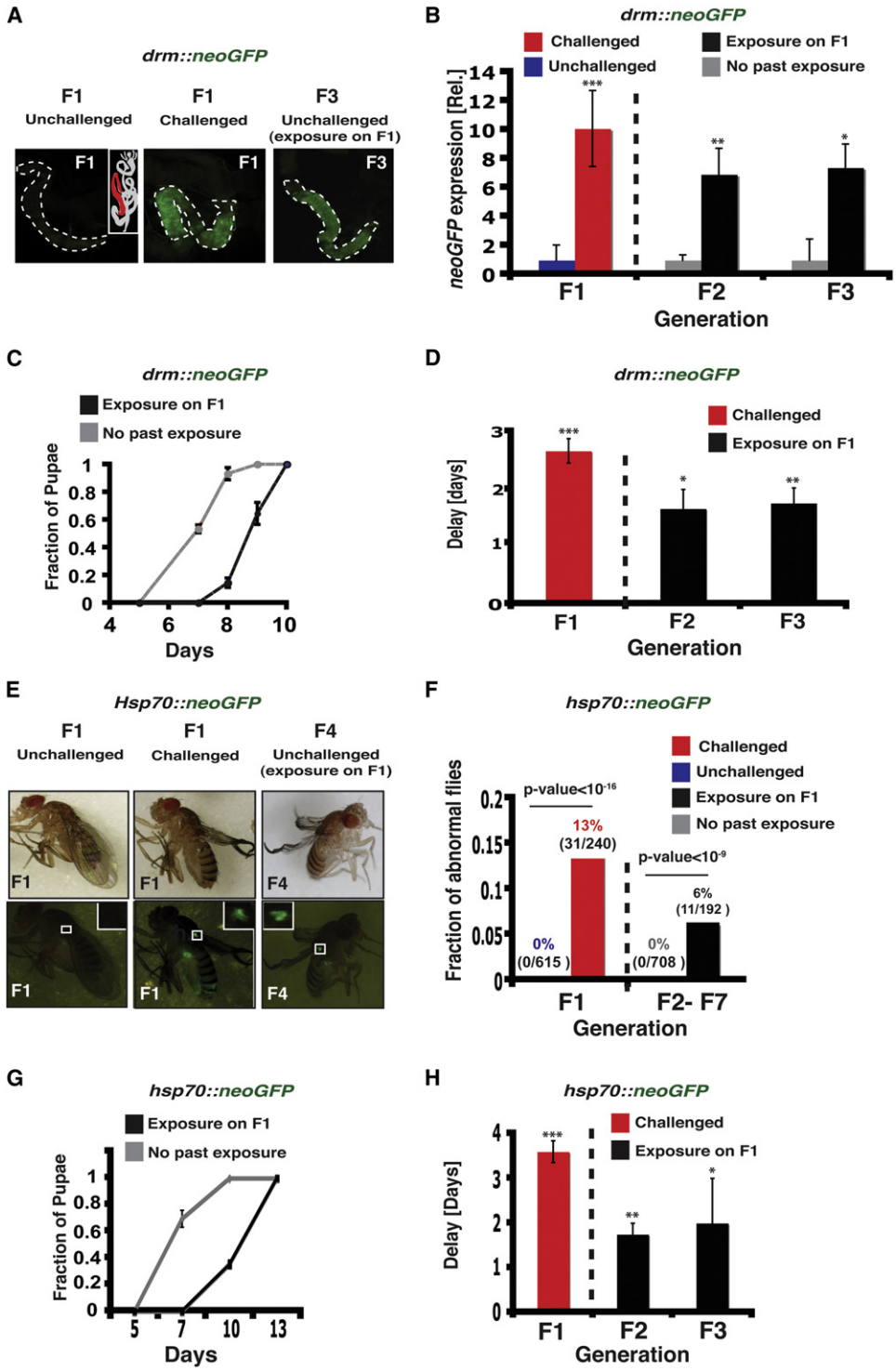

Figure 6. Inheritance of Additional Phenotypes in Other Scenarios of Challenge

(A) Representative images demonstrating elevated expression of *neoGFP* in the midgut of challenged F1 *drm::neoGFP* larvae (center versus left), and in unchallenged F3 offspring following two generations without G418 (right versus left).

(B) Mean *neoGFP* expression (±SE) in the midgut of unchallenged (n = 23) and challenged (n = 15) F1 *drm::neoGFP* larvae, and in unchallenged F2, F3 generations with (n = 12, 19, respectively) or without (n = 16, 20) past exposure on the F1 generation. The dashed lines were drawn to separate results obtained for the F1 generation from those of later generations.

(C) Representative delay in development of unchallenged F2 *drm::neoGFP* larvae with a history of exposure on F1 compared to larvae without past exposure.

express a resistance gene. Although all the studied cases share the same agent of toxicity (G418), the effective stress is expected to depend on the detailed mismatch between the regions that are exposed to G418 and the domains of expression of the resistance gene. The promoter-specific phenotypes that emerged in some of the different setups (e.g., reduced size in *drm::neoGFP* flies and abnormal wings in *Hsp70::neoGFP* flies) are indicative of the potential of this challenge to induce drastic changes in development.

Physiological Deviations from Normal Development

To modify normal development, the challenge has to overcome the mechanisms responsible for the stability (canalization) of the normal patterns. Part of this stability is conferred by the Polycomb system: it maintains the epigenetically repressed state of many developmental regulators, thus preventing abnormal expression of these regulators in ectopic domains. Using the *hairy* promoter as a test case, we showed that the levels of PcG genes are reduced under challenge. This reduction in Polycomb genes is expected to relieve part of their suppressive effect on developmental gene batteries, and may therefore assist the transition into a modified developmental process. A similar suppression of Polycomb function has been reported following injury in the imaginal wing disc of the fly. The latter was mediated by activation of the JNK pathway and was associated with increased incidence of transdetermination events (Lee et al., 2005). We hypothesize that challenge-induced suppression of Polycomb is a general mechanism that reduces barriers against change and facilitates transitions between developmental programs. This type of physiological facilitation might increase the ability of organisms to survive, bridging the timescales between development and evolution (Baldwin, 1896; Schmalhausen, 1949).

The emergence of alternative developmental outcomes within a single generation reflects the degenerate nature of the genotype-to-phenotype transformation (Albrech, 1991; Greenspan, 2001; Rutherford and Henikoff, 2003) and calls for an extension of the strict canalized picture of the developmental process. Although the suppression of Polycomb might be an important facilitator of developmental change, it likely corresponds to a single component in a complex process involving additional regulatory genes and mechanisms that are yet to be identified. We expect that the use of models for presenting artificial challenges will reveal the scope and identity of mechanisms by which developmental systems may deviate from their normal patterns and accommodate rare or unfamiliar challenges by physiological means.

Potential Implications for Evolutionary Diversification

Previous work showed that environmentally induced modifications may develop into stably heritable features after several generations of genetic selection (genetic assimilation) (Waddington, 1953; Gibson and Hogness, 1996; Ho et al., 1983; Suzuki and Nijhout, 2006). Here, we demonstrated immediate nongenetic heritability of multiple developmental features. Other cases of transgenerational epigenetic inheritance of a variety of features have been previously demonstrated in various species (Jablonka and Raz, 2009; Rando and Verstrepen, 2007; Anway et al., 2005; Carone et al., 2010; Cropley et al., 2006), including flies (Cavalli and Paro, 1998; Dorn et al., 1993; Sollars et al., 2003; Xing et al., 2007; Lin et al., 2004; Seong et al., 2011). As in other cases, the heritability that we report is transient. However, it may potentially be stabilized by genetic assimilation, thus leading to stable incorporation of modified features into the developmental program. In addition we cannot exclude the possibility of stabilization via additional epigenetic modifications (Sollars et al., 2003; Ruden et al., 2005) that may render the altered patterns more stable than the normal phenotypes. The chances of genetic or epigenetic stabilization likely increase with the number of generations of persisting phenotypes. In this work we challenged the larvae for only 1 generation, and the inheritance extended for a highly variable number of offspring generations (between 1 and 24 generations). It is plausible that a larger number of challenged generations will extend the duration of occurrence of the modified phenotypes and, hence, the chances of fixation. The aforementioned results therefore call for further exploration of forces that drive the emergence of alternative developmental outcomes (Gilbert and Lloyd, 2000; Rudel and Sommer, 2003) and the potential of these outcomes to contribute to future diversification.

EXPERIMENTAL PROCEDURES

Measurements of Survival Ratios and Duration of Larval Development

Survival ratios were measured as follows: in each experiment the same number of parents were transferred to vials (two females and two to three males per vial) with or without G418 and allowed to lay eggs for the same number of days (2–3 days). Roughly 20–50 progenies are expected to develop in vials without G418 in these conditions. In this experimental setup, F1 larvae were exposed to G418 throughout their larval stages. On days 18–20 the

(D) Mean delay (±SE) in unchallenged *drm::neoGFP* F2 and F3 generations with past exposure on F1 versus no past exposure. Based on three to four replicated experiments. The dashed lines were drawn to separate results obtained for the F1 generation from those of later generations.

(E) Representative images of abnormal wings in *Hsp70*::*neoGFP* adult fly that was challenged during its development (center) and in an unchallenged offspring following three generations without G418 (right). Insets display expression of *neoGFP* at the base of the abnormal wings.

(F) Incidence of wing abnormalities in unchallenged F1 *Hsp70*::*neoGFP* flies (n = 7 experiments, 0 of 615 flies), challenged F1 (200–400 μg/ml G418; n = 7, 31 of 240 flies, 13%), unchallenged offspring within a range of 1–6 generations after last exposure to G418 (F2–F7, n = 6, 11 of 192 flies, 6%), and flies from the same range of generations without past exposure in their ancestors (n = 4, 0 of 708 flies). The dashed lines were drawn to separate results obtained for the F1 generation from those of later generations.

(G) Representative delay in development of unchallenged F3 *Hsp70::neoGFP* larvae with history of exposure on the F1 ancestors compared to larvae without exposure on F1.

(H) Mean delay (±SE) in unchallenged F2 and F3 *Hsp70::neoGFP* larvae with history of exposure on the F1 ancestors. Based on two to four replicated experiments. The dashed lines were drawn to separate results obtained for the F1 generation from those of later generations.

*$p < 0.05$, **$p < 10^{-3}$, ***$p < 10^{-5}$ (Student's t test).

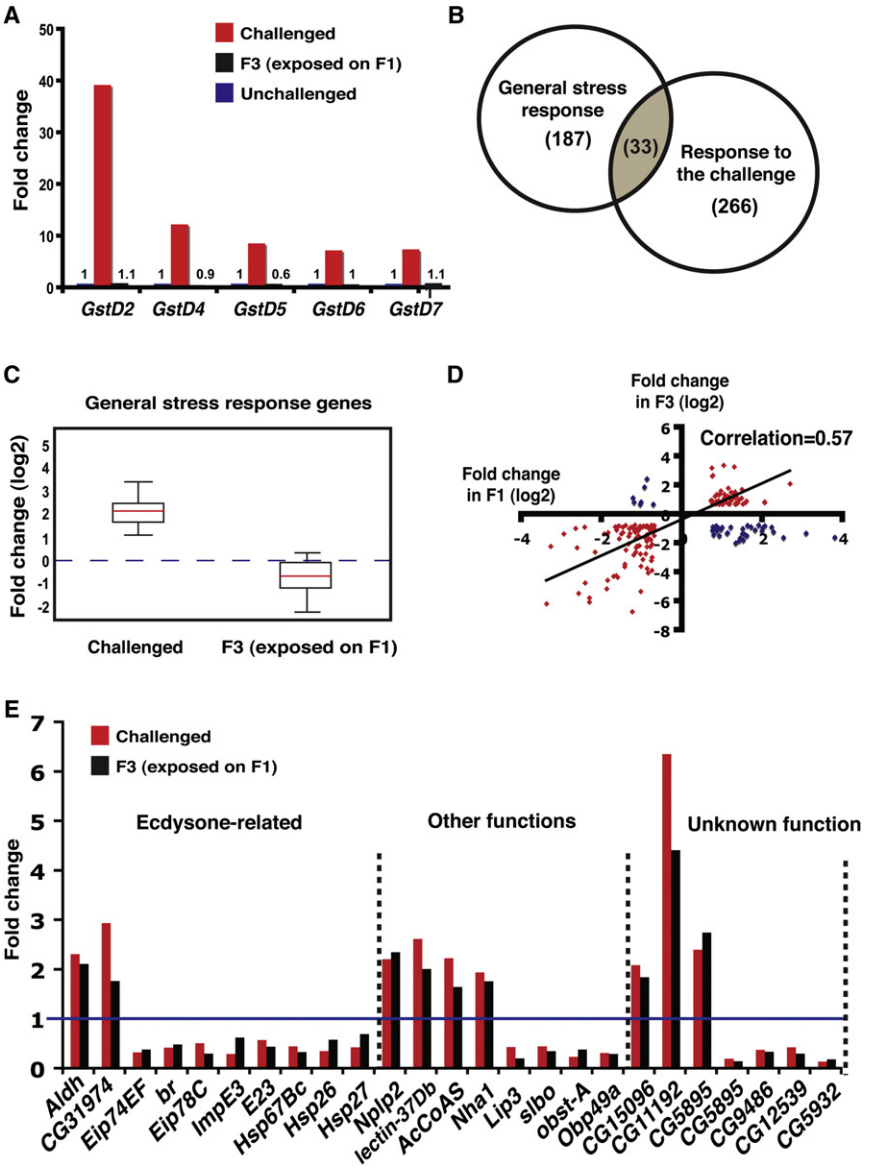

Figure 7. Heritable and Nonheritable Expression Changes in Response to the Challenge

(A) Nonheritable induction of expression of detoxification, *GstD* genes in proventriculi of challenged F1 *hairy::neoGFP* larvae compared to unchallenged larvae (based on microarray data). Data are represented as mRNA levels relative to unchallenged F1 ±SE.

(B) A list of general stress response genes compiled from the literature exhibited a small but significant overlap (12%, $p < 10^{-11}$, hypergeometric test) with the response to the challenge in the proventriculi of *hairy::neoGFP* larvae.

(C) Lack of inheritance of expression changes of the 33 stress genes indicated in (B) ($p < 10^{-22}$, Student's t test).

(D) mRNA expression fold change in the proventriculi of challenged F1 (compared to unchallenged *hairy::neoGFP* larvae; x axis) versus fold changes in unchallenged F3 with a history of exposure in F1 (compared to F3 without a history of exposure; y axis). Shown are genes with fold change above 1.6-fold in both F1 and F3 (189 genes). Red and blue data correspond to positive and negative correlation, respectively.

(E) Example of genes with heritably modified expression.

See Figure S6 and Table S1 as well.

numbers of adult flies in each G418 vial were counted and normalized to the average number of flies in vials without G418 from the same experiment. Survival data on F1 (Figures 2, 3, 4, S2, and S3) correspond to flies heterozygous for both the UAS-*neoGFP* and a specific *GAL4* driver.

Delay in larval development was measured by allowing two females and two to three males to lay eggs for 2–3 days in vials with or without G418. The number of pupae in each vial was counted daily. The integrated number of pupae that formed prior to each inspection time was normalized to the total number of pupae that were formed in the vial at the end of the experiment. Average developmental delay (Figures 2, 4, 5, and 6) was measured by computing the average time gap for pupae formation in different experimental conditions (different environments or genotypes).

Transgenerational Experiments

Three homozygous or heterozygous (balanced) promoter-*GAL4* males and two homozygous UAS-*neoGFP* females were crossed and allowed to lay eggs for 3 days in vials with 400 μg/ml G418 or without G418. F1 flies that were developed from these eggs were collected after 19–20 days from the start of the experiment (4- to 7-day-old adults), and the same number of males and females was crossed again in vials without G418. This procedure was then repeated in subsequent generations, again without G418. Larvae or adult flies from a specific number of generations after last exposure to G418 were compared to larvae or flies from the same generation without past exposure to G418. For most of the transgenerational experiments, flies of the F1 generation were heterozygous for the GAL4 driver and the UAS-*neoGFP* transgenes. For subsequent generations (>F1) we crossed only fluorescent adults (having at least one GAL4 and one UAS-*neoGFP* transgene). The mix of individual genotypes in subsequent generations (>F1) is expected to be the same when comparing larvae with or without past exposure to G418. Potential sampling problems were eliminated by performing a large number of repeats of the inheritance experiment and by verifying the main results using a homozygous *hairy::neoGFP* line, in which case the dosage of *hairy-GAL4* and UAS-*neoGFP* does not change over generations.

Statistical Analyses

All statistical tests were performed using the MATLAB software (MathWorks). Student's t test was used for evaluating the statistical significance of mean values. All cases of significant change were also verified using the Wilcoxon test. Statistical testing of difference between entire distributions (Figures 2H and 4D) was performed using the Kolmogorov-Smirnov test. Significance of overlap between gene sets (Figure 7) was determined using the hypergeometric statistical test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.celrep.2012.03.012.

LICENSING INFORMATION

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