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Genomic Consequences of Background Effects on *scalloped* Mutant Expressivity in the Wing of *Drosophila melanogaster*

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ABSTRACT

Genetic background effects contribute to the phenotypic consequences of mutations and are pervasive across all domains of life that have been examined, yet little is known about how they modify genetic systems. In part this is due to the lack of tractable model systems that have been explicitly developed to study the genetic and evolutionary consequences of background effects. In this study we demonstrate that phenotypic expressivity of the *scalloped*^{E3} (*sd*^{E3}) mutation of *Drosophila melanogaster* is background dependent and is the result of at least one major modifier segregating between two standard lab wild-type strains. We provide evidence that at least one of the modifiers is linked to the *vestigial* region and demonstrate that the background effects modify the spatial distribution of known *sd* target genes in a genotype-dependent manner. In addition, microarrays were used to examine the consequences of genetic background effects on the global transcriptome. Expression differences between wild-type strains were found to be as large as or larger than the effects of mutations with substantial phenotypic effects, and expression differences between wild type and mutant varied significantly between genetic backgrounds. Significantly, we demonstrate that the epistatic interaction between *sd*^{E3} and an *optomotor blind* mutation is background dependent. The results are discussed within the context of developing a complex but more realistic view of the consequences of genetic background effects with respect to mutational analysis and studies of epistasis and cryptic genetic variation segregating in natural populations.

HISTORICALLY genotype × genotype interaction, or epistasis, has been considered of minor consequence with respect to the evolutionary trajectory of a population (Hill *et al.* 2008). However, recent theoretical and empirical investigations have provided a new focus on various forms of epistatic interactions (Hansen 2006). In evolutionary genetics, such interactions are usually estimated in QTL (Pavlicev *et al.* 2008) or linkage disequilibrium mapping (Dworkin *et al.* 2003; Caicedo *et al.* 2004; Steiner *et al.* 2007) studies. However, an alternative approach for studying epistasis is to examine the interactions between new alleles in a population (via mutation or gene flow) and the genetic background in which theses alleles occur (Felix 2007) and, in particular, to determine the evolutionary consequences of these "genetic background effects" (Dworkin 2005a; Masel 2006). One example of an interaction between allelic variants and genetic background is the phenomenon described as cryptic genetic variation (CGV). Phenotypes that are otherwise invariant in natural populations can often be sensitized to reveal underlying phenotypic variation, via mutations of large effect or an external environmental stressor (Waddington 1952). It has been well demonstrated that this revealed variation has a genetic basis and that natural populations are segregating alleles that contribute to the expressivity of these phenotypes (Waddington 1952; Bateman 1959; Milkman 1962; Gibson and Dworkin 2004). These results suggest that allelic variation within genetic networks can modulate the otherwise deleterious consequences of mutant alleles (Dworkin 2005a). However, as it is unclear if they are having any other phenotypic effect (in the absence of the perturbation), it is unknown how selection acts on these variants and how they are maintained in populations. In addition, there is evidence that some modifiers of allelic function are the result of naturally occurring polymorphism of the gene under study. Methods such as association mapping can be used to help identify novel alleles of a particular modifier gene (Dworkin *et al.* 2003). Thus, these background effects contribute to cryptic genetic variation for phenotypes and may represent an important source of genetic

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Arrays have been submitted to the GEO database at NCBI as series GSE13779.

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variation in natural populations (Hansen 2006; Barrett and Schluter 2008; Le Rouzic and Carlborg 2008).

Interest in how genetic background modifies allelic expressivity is not limited to evolutionary questions, but is also a consideration for functional genetic analysis (Nadeau 2001, 2003). One of the basic tools used for the study of development is the analysis of loss-of-function (LOF) mutations to determine what, if any, function a given gene may have. In particular the analysis of LOF mutations can establish whether a gene is necessary for a particular developmental event or regulation of other genes. Developmental genetic analyses often use allelic series ranging from weak hypomorphic to null (amorphic) mutations to study specific aspects of the gene structure/function relationship, and it is clear from the advances in development over the past several decades that this has largely been a successful approach (Lewis 1978; Nusslein-Volhard and Wieschaus 1980). While many genetic screens are carried out in otherwise isogenic backgrounds, subsequent analyses often utilize alleles of a gene that come from a number of studies, each isolated in a different genetic background. Unfortunately the consequences of the background effects are rarely explicitly addressed and thus remain a confounding effect in the analysis, and subsequent interpretation of, the phenotypes. This concern may be particularly acute for the analysis of functional epistasis.

A number of studies have established that the genetic background effects of different wild-type strains are pervasive for a wide variety of common model developmental systems such as homeosis (Gibson and van Helden 1997), ocular retardation in mice (Wong *et al.* 2006), cell signaling and determination (Threadgill *et al.* 1995; Polaczyk *et al.* 1998; Dworkin *et al.* 2003), and the establishment of neurogenic clusters (Dworkin 2005a). In particular it has been established that the phenotypic consequences of the background effects can be as substantial as induced mutant modifiers (Gibson *et al.* 1999; Atallah *et al.* 2004). To date, it remains unclear if the loci that underlie the background effects are genes that would otherwise be identified in sensitization screens or are a unique set. Nor is it clear whether the background effects are due to a small number of modifier loci (Gibson *et al.* 1999) or to a relatively large number of allelic variants with small effect size (Polaczyk *et al.* 1998; Dworkin *et al.* 2003).

We recently introgressed 50 mutations into each of two wild-type strains to study their effects as heterozygotes on wing shape (Dworkin and Gibson 2006). In addition to its effect on wing shape, the *scalloped*^{E3} (*sd*^{E3}) mutation demonstrated a substantial wing reduction phenotype in the hemizygous (and homozygous) state. Interestingly, the phenotypic effects of the *sd*^{E3} allele varied depending on which of the two different wild-type strains the mutation was observed in. *Sd* encodes a TEA class transcription factor that forms a heterodimer with Vestigial (Vg) and together act as a cofactor for numerous transcription factors (Halder *et al.* 1998; Paumard-Rigal *et al.* 1998; Simmonds *et al.* 1998), in a process that is necessary and sufficient to confer wing determination (Halder *et al.* 1998; Guss *et al.* 2001; Halder and Carroll 2001). LOF alleles of both *vg* and *sd* lead to varying degrees of wing reduction, depending upon allele severity (Srivastava *et al.* 2004). It has also been demonstrated that the stoichiometry of Sd and Vg in the developing wing disc is important for the proper development of the wing (Delanoue *et al.* 2004; Legent *et al.* 2006).

In this study, we introduce the *sd* background effect as a model system to study genetic background effects. Previous work suggests both simple and complex genetic architectures: a major QTL modifies the *Ultrabithorax*^{1} homeotic phenotype (Gibson *et al.* 1999), while modifiers of an *Egfr* gain-of-function allele suggest that the architecture of this photoreceptor determination phenotype was more likely due to many alleles of small effects (Dworkin *et al.* 2003). We ask here whether microarrays are a fine enough tool to dissect gene expression changes mediating background differences in mutant expressivity and hence may give a more global view than QTL mapping. In particular we use genomewide expression data to test between several alternative models of how genetic background modifies the *sd* phenotype: (1) Background effects are mediated independent of quantitative differences in transcription; (2) the *sd* mutation enhances background-specific quantitative differences in transcription, mediating the observed phenotypic differences; (3) the background effects involve a set of genes that overlap only partially with the genes that are differentially expressed between mutant and wild type, with quantitative differences in transcription that correlate with variation for the *sd* phenotype; and (4) the background effects involve a different set of genes from those that mediate the main effects of the mutant *sd* allele. Our results are broadly consistent with the latter two models.

Concordant with earlier studies, we demonstrate that LOF mutations in *sd* appear to lead to a retardation of cellular growth and metabolism, while the background differences are associated with changes in the expression of a number of key developmental regulators in the wing, largely consistent with the final model (4). However, for a small number of genes, changes in gene expression patterns mirror the observed morphological phenotypic effects in terms of mutant expressivity, consistent with a common subset that varies quantitatively with transcript abundance. Finally, we provide evidence that *bi/omb* interacts epistatically with *sd* to contribute to the wing reduction phenotype in a background-dependent manner and that a region linked to the *vg* locus is associated with the background effect. We discuss these results within the framework of the genetic architecture of background effects and the role of such epistatic interactions in the maintenance of genetic variation.

MATERIALS AND METHODS

Fly strains: The X-linked *sd*^{E3} mutant used in this study was originally obtained from the Bloomington Stock Center. This mutant allele is caused by a P{*w*[E] ry[+t7.2]=wE} transposon located in the third intron of the gene (Inamdar *et al.* 1993) and is unlikely to affect resulting protein activity. This mutant allele was introgressed into two lab wild-type strains, Oregon-R and Samarkand, both marked with *white* (*w*), as described in detail in Dworkin and Gibson (2006). At least 20 generations of backcrossing of the mutation was performed in each background prior to the analyses discussed in this study. In addition, the P{GawB}*bi/omb*^{md653} allele (obtained from Bloomington) was introgressed into both the Samarkand and the Oregon-R backgrounds and was recombined onto the chromosome with *sd*^{E3} (in the appropriate background) for phenotypic analysis. Both wild-type strains were genotyped for a number of common inversion polymorphisms in *Drosophila melanogaster* and appear to be homotypic for the common chromosomal arrangements.

Markers: To assess the extent of the efficacy of the introgression of the *sd*^{E3} allele into each genetic background we screened previously developed markers found at FLYSNP (http://flysnp.imp.ac.at) for polymorphism between Samarkand and Oregon-R. Following >20 generations of introgression into each of Samarkand and Oregon-R we observed no evidence of residual segregating sites across 20 markers. In addition, markers as close as 3 cM away from *sd* were successfully introgressed from each of the wild-type lines, which sets an upper boundary of 6 cM for the region linked to the *sd*^{E3} allele.

Transcriptional profiling: *RNA isolation protocol:* Larvae for the relevant genotypes were reared at 25° in bottles on standard media. Wing imaginal discs from wandering third instar were dissected out of larvae in PBS on ice and then transferred to RNAlater. RNA was stored at −80° until purification. Two replicate sets of dissections were performed, collecting ∼40 mature third instar wing discs per replicate. RNA was extracted and purified using the QIAGEN (Valencia, CA) RNAeasy kit, with a DNase1 digestion to remove any traces of contaminating DNA prior to cDNA synthesis.

DGRC arrays: *RNA amplification and labeling:* Given the small amount of mRNA that can be obtained from imaginal discs, a linear amplification (Agilent low-yield RNA amplification kit) was used for all samples. Four replicate amplifications were performed for each genotype, using 500 ng/replicate. The remaining protocol represents a modified version of TIGR protocol (Passador-Gurgel *et al.* 2007). After clean-up, the replicates were pooled and split, followed by an overnight cDNA synthesis and indirect amino-allyl labeling. After dye incorporation and clean-up, the relevant samples were mixed, dried down, and resuspended in hybridization buffer.

Experimental design for array hybridization: A balanced incomplete block (where block equals array) design was used in a full loop configuration with dye swapping. This design avoids confounding any variables with dye effects. Twelve two-channel hybridizations were performed, with 6 replicate hybridizations per treatment (3 per treatment per dye). Hybridizations were performed for 16 hr at 42°, using a MAUI Mixer (BioMicro Systems).

Arrays: The Drosophila Genome Reseach Center (DGRC) v1 arrays were used in this study. These arrays consist of 15,552 features spotted with amplicons from genomic DNA for 14,151 primer pairs, representing 13,801 annotated amplicons for the release 4.1 genome. These correspond to 11,880 unique genes, with ∼13% redundancy genomewide. Pre- and posthybridization procedures followed the DGRC indirect-labeling protocol. The arrays were scanned on a Perkin–Elmer scanner.

Analysis: *Extraction of microarray data:* To extract feature information from microarray images, spot segmentation was performed using the connected component algorithm in UCSF Spot (Jain *et al.* 2002). These results were compared with the histogram-based approach and found to have similar, though somewhat more reliable, results (not shown).

Global normalization and gene-specific models: Log_2-transformed signal intensities were normalized using the linear mixed model (GLMM) approach (Jin *et al.* 2001), adjusting for dye (fixed), array, and array × dye (random) effects. In addition, we also examined the effects of normalization when print-tip/subarray was included as a fixed effect (including interactions between print-tip, array, and dye). While it was clear that including print-tip in the normalization model significantly improved the fit, it had minimal effects on the gene-specific models (not shown) since almost all probes on the array are unique and spotted in the same position, and it was not used for the analyses presented here. Results following a robust (median-based) normalization were similar to those from the mixed model (not shown).

Residuals from the global normalization were then used in the following spot (transcript)-specific mixed models:

$$\mathrm{spot}_{ijklmn} = \mu_i + G_{ij} + B_{ik} + GB_{i(jk)} + D_{il} + A_{im} + \varepsilon_{ijklmn}.$$

For the *i*th spot, μ_i is its intercept, G_{ij} is the *j*th genotype (*sd* or wild type, wt), B_{ik} is the *k*th background (Oregon-R or Samarkand), and D and A model the spot-specific dye effect and array variance, respectively. All factors except array and the residual error, ε were treated as fixed effects. Analysis was performed using Proc Mixed in SAS (v9.1).

To address the multiple-testing problem inherent in microarray analysis the false discovery rate method of Storey (Storey and Tibshirani 2003) was implemented in the *q* value library of R V2.3 (Ihaka and Gentleman 1996). Unless otherwise indicated, a *q* value of ≤0.01 was always used, implying that <1% of genes whose expression is identified as significantly different represent false "hits."

Gene ontology analysis: GOTREE (Zhang *et al.* 2005) and Expander (Shamir *et al.* 2005) were used to ask whether genes that are differentially expressed within treatments are overrepresented (relative to all of the genes on the array) for gene ontology (GO) categories, applying a sequential Bonferroni correction method. Care must be used in interpreting the number of categories deemed significantly overrepresented as GO categories are not independent of one another. However, this method can still provide a broad picture as to the groups of genes that show differential expression.

In situ hybridization protocol: Drosophila imaginal tissues were dissected from larvae in phosphate-buffered saline and placed into the standard fixative. Digoxigenen-labeled antisense RNAs were prepared and *in situ* hybridizations were done essentially as in Tautz and Pfeifle (1989) for the genes *vg*, *sd*, *Dll*, *omb*, *wg*, and *salm*.

Marker genotyping: Sequences were obtained using standard Sanger sequencing methods. To follow the *vg* indel polymorphism in the quadrant enhancer, the primers F-ACGGATACA AGTGCAAGGACACAC and R-TAGTGCGGTCCTGCACAGA GAAA were used. F_2's and F_7 intercrosses were produced, and DNA was extracted using "squish" preparations (Gloor *et al.* 1993). F_2 and F_7 individuals were phenotyped, and the extremes (∼8% of each tail) of the distributions (long- and short-winged individuals) were used to perform bulk segregant analysis.

RESULTS

A major-effect modifier of expressivity of the *sd*^{E3} mutation maps near *vestigial*: Introgression of the *sd*^{E3} mutation for >20 generations into each of two standard

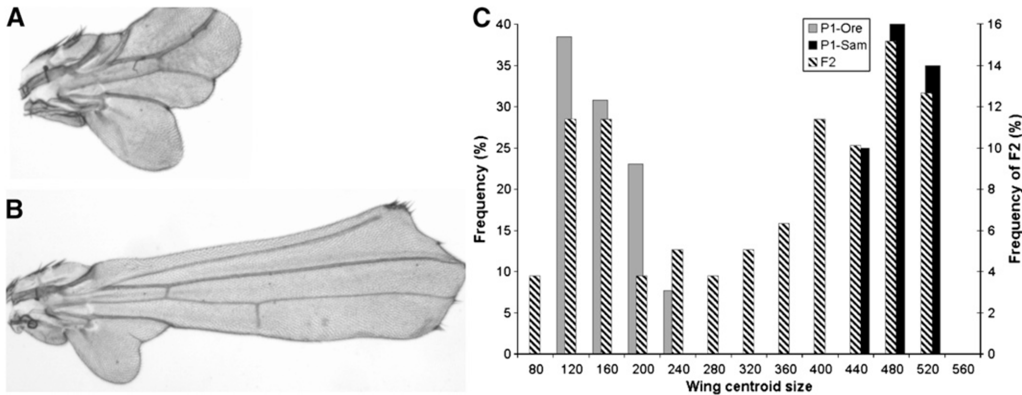

FIGURE 1.—Phenotypic consequences of genetic background on the expressivity of the sd^{E3} allele in the wing. In the Oregon-R background (A) this is similar to severe hypomorphic alleles of *sd*, whereas in the Samarkand genetic background (B) the effect resembles a weak to moderate allele. (C) The distribution of the wing sizes for the sd^{E3} allele in each wild-type genetic background is completely nonoverlapping. The distribution of the F_2 population shows clear evidence of bimodality, consistent with at least one modifier of large effect segregating between the two wild-type backgrounds.

lab wild-type backgrounds, Samarkand and Oregon-R, results in substantial phenotypic differentiation both in hemizygous males and in homozygous females (Figure 1A). Whereas normal wings of these two strains differ only subtly in the shape of the posterior intervein region, sd^{E3} Oregon-R wings are reduced throughout the wing blade, while sd^{E3} Samarkand wings remain elongate with substantial loss of tissue from the anterior and posterior margins. Consequently, the distributions of sd^{E3} wing size in these two genetic backgrounds are completely nonoverlapping (Figure 1C). These effects are reminiscent of the difference between weak and severe hypomorphic alleles for this gene (CAMPBELL *et al.* 1992; SRIVASTAVA *et al.* 2004). Using 18 anonymous molecular markers spread across the genome, we found no evidence of regions that were not completely introgressed, and the region linked to the sd^{E3} allele must be no greater than 6 cM, representing <1.6% of the euchromatic X chromosome according to the release 5 assembly (∼0.3% of the euchromatic genome). Despite the small size of this residually linked region, the formal possibility exists that the background effect is due to other loci linked to the sd^{E3} allele in at least one of the two strains. However, as discussed below, there was no evidence for any genetic effect on the X chromosome, thus eliminating this possibility.

Generation means analysis implies that a considerable proportion of the background effect is likely due to a single major-effect modifier whose influence on wild-type wing pattering is cryptic. The mean wing size of F_1 progeny of Samarkand by Oregon-R sd^{E3} mutant flies is biased toward that of Samarkand mutants, implying that the Oregon-R alleles that lead to strong wing reduction are largely recessive (not shown). More interestingly, the distribution of wing size observed in the F_2 individuals shows a clear bimodality (Figure 1C). This pattern is most clearly explained by at least one modifier of large effect in addition to smaller environmental and genetic contributions.

Given the intimate functional relationship between Sd and Vg in wing patterning, we tested whether allelic variation in either of these genes may be responsible for the major modifier effect. Natural allelic variation at *scalloped* contributing to the background effects was excluded as a possibility given that the phenotype is observed in the hemizygous males for sd^{E3}, where there is no natural allele of *sd* to interact with the sd^{E3} mutation. In addition, F_1 male progeny of reciprocal crosses between strains carrying the sd^{E3} and the alternate wild-type strain do not show any significant difference in wing size. Since *sd* is X-linked, a major effect of this locus would have been expected to reproduce the background-dependent size difference. In addition, no markers segregating on the X chromosome between Oregon-R and Samarkand were associated with the background effects (not shown).

Linkage of background modifier to vg region: To test for a contribution of *vg*, the sequences of the functionally characterized *vg* regulatory enhancers were generated from both the Oregon-R and the Samarkand strain. The most notable polymorphism detected was a 40-bp complex deletion observed in the quadrant enhancer in Oregon-R. We utilized this deletion as a marker to determine if *vg* itself was a modifier of the *sd* phenotype with respect to the background effect. A possible contribution of *vg* was suggested by linkage with the Oregon-R deletion polymorphism in a small cohort of F_2 flies derived from a cross of Sam sd^{E3} to Oregon-R sd^{E3} flies. The short winged phenotype showed a perfect association with the *vg* deletion allele in 24 of 24 individuals, all of whom were homozygous for the Oregon-R deletion polymorphism. To validate this association, the contribution of *vg* was tested following phenotype-based introgression of the long-wing (Samarkand-derived) phenotype into the short-wing (Oregon-R derived) background for seven generations. In these phenotype-based introgression-selection lines the deletion allele in the vg quadrant enhancer derived from Oregon-R was displaced by the Samarkand allele in two independent sets of introgressions, further supporting close linkage of the modifier to *vg*. However, genotyping of a sample of phenotypically extreme F_7 backcross individuals (the 5% shortest and longest wings from the sample) for the *vg* insertion/

TABLE 1

Lack of independence between *vg* insertion/deletion polymorphism and genetic background effect for bulk segregant analysis using phenotypic extremes for a set of F_7 progeny

	Short wing	Long wing
i/i	3	3
i/d	12	15
d/d	28	8
χ^2	8.18 ($P = 0.016$)	

i, insertion allele from the SAM background; d, deletion allele from ORE.

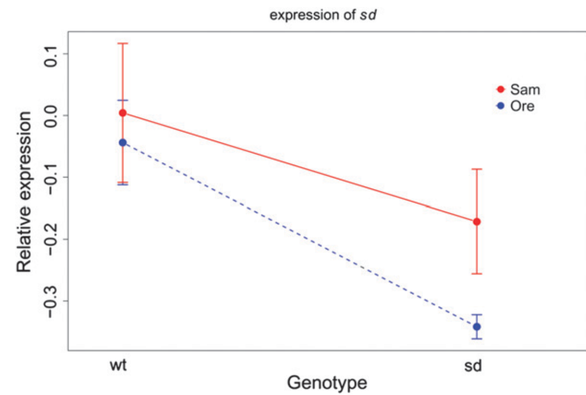

Figure 2.—The expression of the *sd* transcript is reduced in sd^{E3} mutants, independent of genetic background. Relative abundance of *sd* transcript is reduced in sd^{E3} individuals as measured using microarrays. There is no statistical support for an interaction between genetic background and the mutation with respect to the abundance of *sd* transcript, and results from quantitative RT–PCR also failed to support an interaction (not shown). Error bars are ±1 SE.

deletion polymorphism revealed incomplete linkage (Table 1). The evidence does not support the hypothesis that *vg* is the large-effect modifier locus, although it is either linked to or interacts epistatically with it.

The genetic background effect is mediated immediately downstream of *sd*: To determine whether the background effect is mediated upstream or downstream of Scalloped activity, we performed a series of *in situ* hybridizations to third instar imaginal discs at the stage when patterning of the future wing blade occurs. The distribution of *sd* transcript in sd^{E3} mutant wing discs is reduced relative to wild type, but there is no evidence of a qualitative difference in expression of *sd* between Samarkand and Oregon-R mutant discs (not shown). Nor was a quantitative effect of genetic background on *sd* expression detected by the microarray analyses described below (Figure 2). While the results of Figure 2 are suggestive of a background effect on *sd* expression, further analysis using quantitative real-time PCR did not support this (not shown). This result suggests that the background modifier likely acts downstream of, or parallel to, *sd* in the wing patterning network (Halder and Carroll 2001).

As an initial test of the models proposed in the Introduction to explain the genetic background effect, we examined the expression of several known *sd*-dependent genes. As shown in Figure 3, several of these developmental patterning genes show changes in the spatial distribution of mRNA consistent with the third proposed model, where transcriptional changes are proportional to the observed *sd* wing phenotypes in each genetic background. For example, in the Oregon-R sd^{E3} background, expression of *vg* is substantially reduced, similar to the effects of a strong hypomorphic allele of *sd* (Figure 3). However, in the Samarkand background, there is a relatively modest change in the distribution of *vg* transcript, relative to wild type. This is consistent with microarray results (below) that found only weak evidence for a difference in *vg* transcript abundance between SAM wild type and Sam sd^{E3} (Diff $= -0.19 \log_2$ units; $t = 2.3$; $P < 0.05$) that would not hold up to multiple comparisons. Similar results were observed for *Dll* transcripts (Figure 3, row 2).

One of the most dramatic differences was seen for *bi/omb*, which seems to have reduced expression in the wing pouch in particular in the Oregon-R background, while the expression in the rest of the wing disc seems normal (Figure 3, row 3). Transcripts of *salm* show almost a wild-type pattern of expression in the Samarkand *sd* genotype, but in the Oregon-R sd^{E3} genotype, the expression is reduced along the future proximal–distal axis, resulting in an oval (with the long axis of expression occurring along the anterior–posterior axis) instead of the more rectangular wild-type expression (not shown). Expression of *wg* along the margin is only partially lost in the Samarkand background, but it is almost completely absent in the Oregon-R background (Figure 3, row 4).

These results indicate that targets of Scalloped transcriptional regulation are differentially expressed, but they do not establish that the effect is direct. It may result from differential modulation of Sd activity by cofactors with variable activity or reflect indirect consequences of other target genes. In either case, cryptic variation for wing shape involves differential expression of a large number of genes downstream of *sd* activity.

To confirm that one of these differences in target gene expression is functionally important for the wing phenotype, we constructed double-mutant combinations of the omb^{md653} and sd^{E3} alleles in both the Samarkand and Oregon-R backgrounds. In the hemizygous state, the omb^{md653} allele shows delta-like venation defects with incomplete penetrance (Dworkin and Gibson 2006), while the central distal wing pouch is missing in homozygotes (Grimm and Pflugfelder 1996). *Trans*-heterozygous females for omb^{md653} and sd^{E3} demonstrate the same incomplete penetrance for the venation

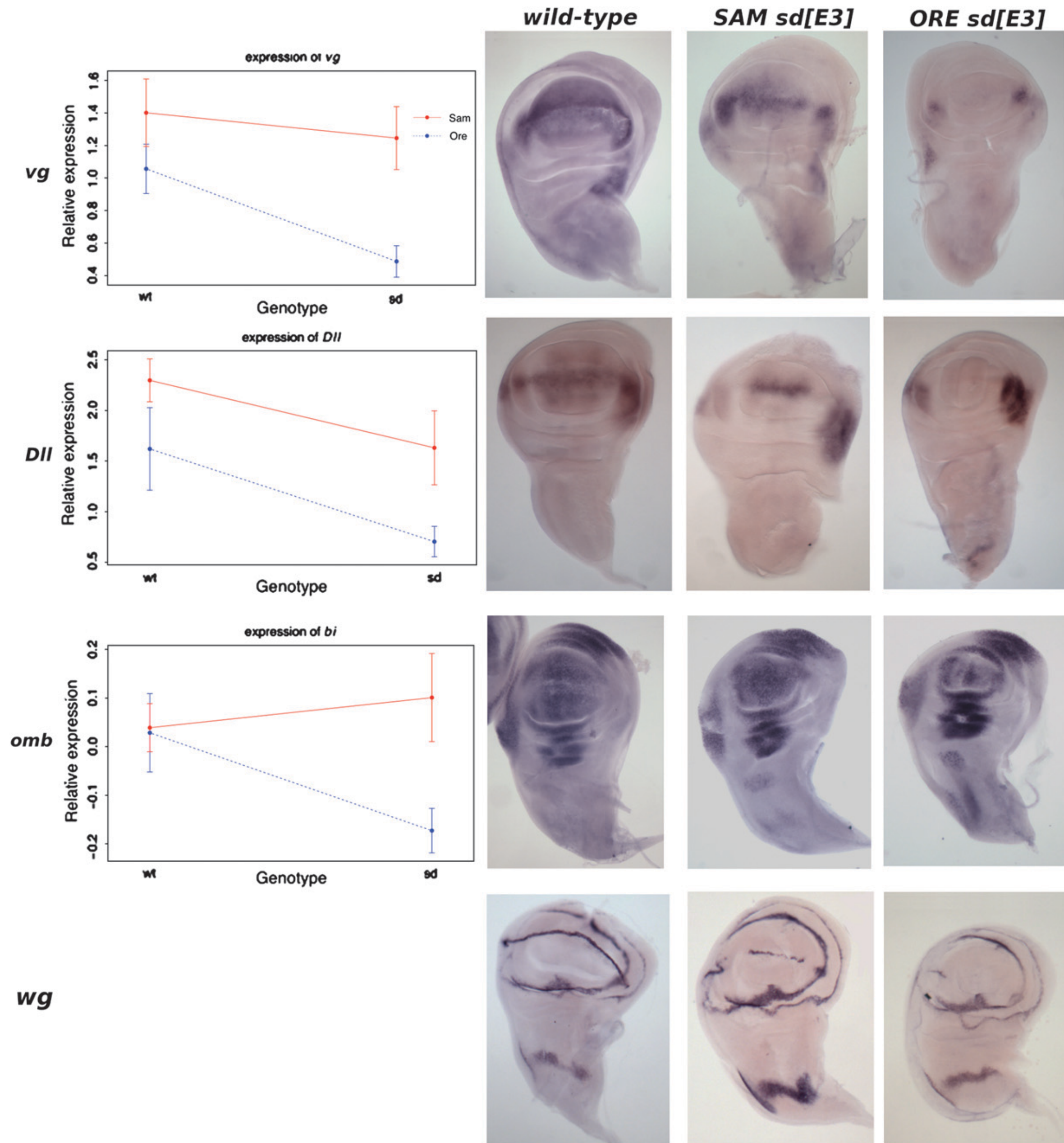

Figure 3.—Background-specific spatial and quantitative patterns of gene expression in Sd-regulated genes. The left column shows the reaction norm plot of relative transcript abundance as monitored using the DGRC arrays for each of four genes (*vg*, *Dll*, *omb/bi*, and *wg*). Consistent with the patterns of phenotypic expressivity between genetic backgrounds, we observed a significant decrease in gene expression, as monitored with *in situ* hybridization of wing imaginal discs, as well as from the microarrays. Row 1 shows that expression of *vg* transcript is reduced in a genetic background-specific manner. While there is no difference between the two wild-type genetic backgrounds, they do differ significantly under sd^{E3} (±1 SE). Consistent with this, the spatial domain of *vg* transcript is reduced in the ORE sd^{E3} background relative to SAM sd^{E3}. Both ORE sd^{E3} and SAM sd^{E3} show spatial restriction relative to wild-type expression patterns for *vg*. Similar patterns of expression were observed for a number of genes including *Dll* (row 2) and *omb/bi* (row 3). Interestingly, two candidate genes (*wg*, row 4; and *salm*, not shown) show clear expression differences between genetic backgrounds with sd^{E3}, but with little evidence for expression differences in the array data.

defects, but have otherwise wild-type wings. The recombinant double-mutant combination of sd^{E3} and omb^{md653} in the Samarkand background results in wings that are phenotypically similar to those observed for the single sd^{E3} mutant in the Oregon-R background (Figure 4A). However, in the Oregon-R background the sd^{E3} omb^{md} double-mutant combination is qualitatively indistinguishable from the Oregon-R sd^{E3} single-mutant phenotype (Figure 4B). This result demonstrates that *bi/omb* behaves as an enhancer of the sd^{E3} phenotype

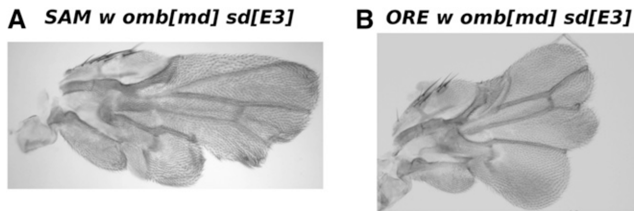

FIGURE 4.—Background-dependent genetic interaction between mutations in *omb* and *sd*. Double-mutant combinations between the *omb*^{md} and *sd*^{E3} alleles were made in both the Samarkand and the Oregon-R genetic backgrounds. (A) In the Samarkand background the double-mutant combination (observed in hemizygous males) shows a strong enhancement of the *sd*^{E3} phenotype (compare with Figure 1B). However, in the Oregon-R background (B) the double-mutant combination is indistinguishable from the qualitative and quantitative range of ORE *sd*^{E3} single mutants (compare with Figure 1A).

only in the Samarkand background, suggesting that the ordering of epistatic interactions requires careful control of genetic background, as there are segregating modifiers of such effects in natural populations, including standard lab wild-type lines.

Microarray analysis of mutant *sd*^{E3} and wild-type wing imaginal discs: While the results presented above suggest that a subset of known *sd*-dependent genes demonstrates transcriptional differences consistent with the third proposed model, we tested the relative contributions of these models to the observed differences in expression of the entire wing transcriptome, examining the joint contribution of genotype and genetic background. Using microarray analyses of wild-type and *sd*^{E3} wing imaginal discs in both genetic backgrounds, a mixed linear model was fit to simultaneously estimate the effects of mutant, background, and interactions between these two factors for each element on the array. Six technical replicates for each of the four genotypes, with balanced dye swaps of labeled RNA, were hybridized to DGRC arrays that contain spotted amplicons for transcripts for ~88% of known and predicted genes from the V4.1 release of the *D. melanogaster* genome. Given the design and statistical analysis used, we could detect significant differences for genes with as low as 1.1-fold differences. Since most transcripts are expressed only in a small subset of wing imaginal disc cells, such differences are likely to reflect a wide range of fold changes of gene expression in specific subsets of cells.

The largest effect on transcript abundance was observed for the comparison of mutant against wild-type discs. Specifically, 1230 array features were deemed to be differentially expressed between *sd* and wild type at a false discovery rate (FDR) of 0.01 (implying that ~12 spots identified as significant are false positives). These 1230 features correspond to 1155 unique genes. For those genes with at least two independent probes the correlation between expression was $r = 0.71$. The high-dimensional microarray expression data are summarized in volcano plots (Figure 5) of the expression differences (on a $\log_2$ scale) on the *x*-axis with a measure of the magnitude of the statistical association between expression differences and treatment effect on the *y*-axis. In general, genes that are deemed to be differentially expressed show the largest fold changes as well as statistical significance. Comparing the *sd*^{E3} mutant discs to wild-type wing discs, there is a marked asymmetry in the magnitude of the association for genes whose expression is altered in the presence of *sd*^{E3}. This asymmetry is characterized both by underrepresentation of genes that are upregulated in the *sd* mutant condition and by an increase in variance for the mutant class (not shown).

Tests for overrepresentation of differentially expressed genes according to gene ontology categories generally highlight a reduction in cellular growth and metabolism in the mutant discs. The reduction in number of cells due to loss of function of *sd* activity thus appears to be due to a retardation of growth. These results are consistent with previous observations of the role of modulators of cellular growth and cell cycle progression (SRIVASTAVA and BELL 2003; DELANOUE *et al.* 2004; LEGENT *et al.* 2006) for wing patterning, but our results do not establish whether Sd directly regulates such genes. Additionally, as shown in Table 2, a subset of the genes that are differentially expressed are known developmental regulators of wing development, including a number of components of Notch and Wingless signaling, as well as downstream targets of Sd. Several genes that are normally expressed at the wing margin [such as *wnt-6* and the *e*(*spl*) transcripts] are also downregulated in *sd* mutants.

Background effects on expressivity of the sd^{E3} *mutation:* As expected, the genetic background was found to have a greater impact on gene expression in the mutant rather than the wild-type discs. Contrasting transcriptional profiles from *sd*^{E3} wing imaginal discs in the Oregon-R and Samarkand backgrounds, 363 spots representing 324 unique genes were called significant at the FDR *q* value <0.01. Surprisingly, the overall fold changes in expression between background effects were substantially greater than those observed for the *sd vs.* wild-type comparison.

Table 2 shows that several GO categories corresponding to developmental functions, as opposed to cell growth and metabolism, are overrepresented in these background-specific genes. This result suggests that while many of the expression differences associated with *sd* are in genes associated with the basic "worker" cellular machinery, the modulation of phenotypic expressivity is likely a consequence of the developmental "bureaucrats" such as transcription factors and signaling pathways.

Contrasting Samarkand and Oregon-R wild-type wing discs, 245 spots representing 189 genes were found to be differentially expressed at the less stringent *q*-value cutoff of 0.05 (and just 138 genes at $q < 0.02$). This

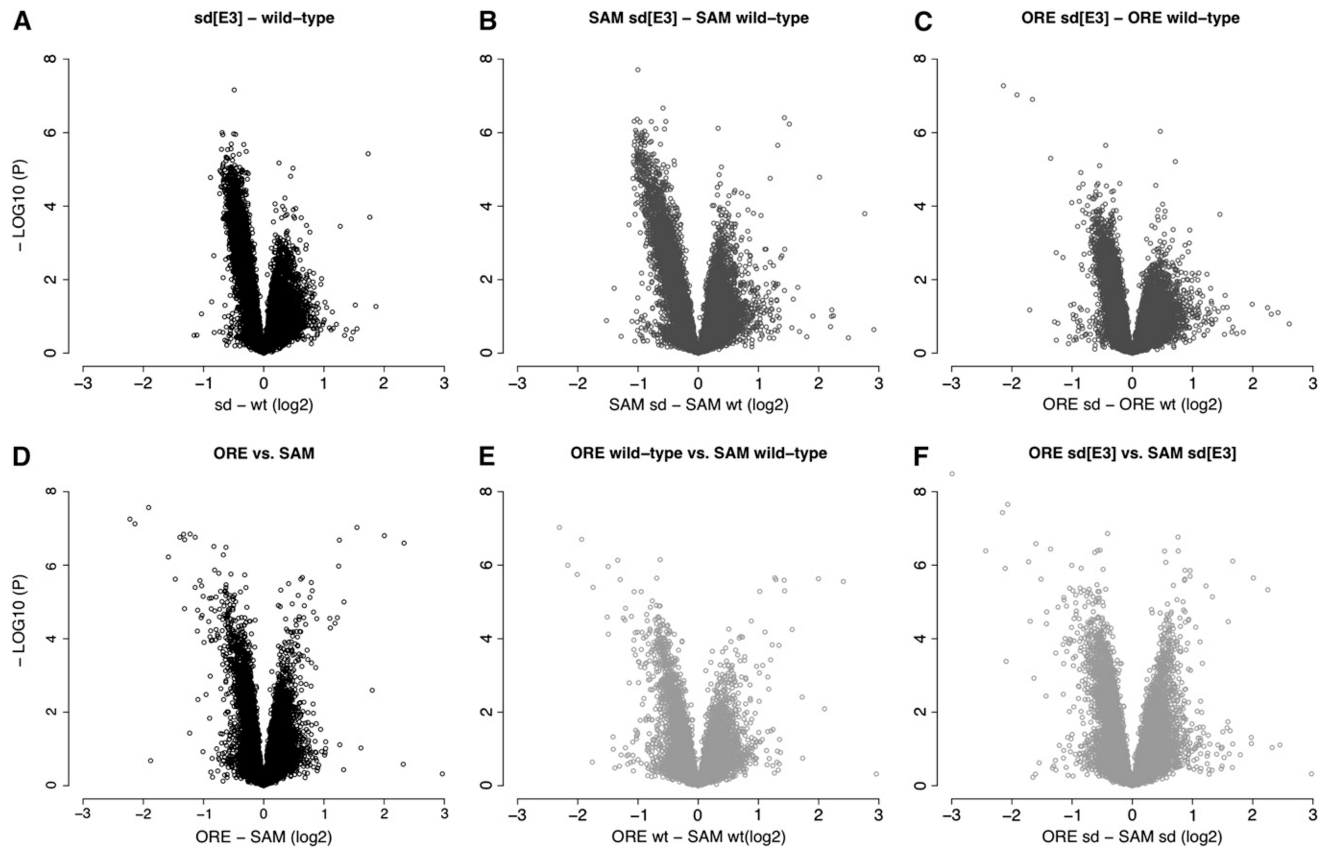

FIGURE 5.—Consequences of the sd^{E3} mutation on the wing imaginal disc transcriptome are background dependent. The majority of differentially expressed genes between sd^{E3} and wild type are the result of a reduction of expression in the sd^{E3} mutant condition (A–C), as demonstrated by the strong asymmetries. These effects appear to be background independent (B *vs.* C). In contrast (D–F), fewer genes show evidence for differences in expression between backgrounds; however, the magnitude of the differences tends to be larger than in the comparison of wild type to sd^{E3}.

represents <2% of the Drosophila genome, but between 5 and 10% of the wing disc transcriptome, consistent with estimates of genotype-specific gene expression in whole flies (JIN *et al.* 2001; MEIKLEJOHN *et al.* 2003). As can be seen in Figure 5E, a number of these transcripts show substantial changes of more than twofold in the magnitude of expression. Sixty-two of these genes were common background-effect genes (*i.e.*, showed differences in comparisons of both the wild-type and the mutant discs), while only 10 of this common list showed evidence for a genotype × background interaction. Thus, at least one-quarter of the genes that differ between wild-type backgrounds continue to show differences in the mutant discs. At $q < 0.05$, 280 features representing 228 unique genes were deemed to show such a genotype × background interaction, further implying that there is considerable background-specific misregulation of gene expression in the mutant discs.

The transcriptional architecture of genetic background effects: As one approach to addressing how genetic background and the mutation interact to alter the genomic transcriptional profile, we wanted to address how similar changes in expression would be either across genetic background or across mutant and wild-type genotypes.

When ORE sd^{E3} is contrasted with SAM sd^{E3} (Figure 5F), 334 genes are differentially expressed, of which 62 are shared with the related comparison with wild type (SAM *vs.* ORE, 218 genes differentially expressed). Thus 62/491 (12.6%) of all of the differentially expressed genes between ORE and SAM are expressed across treatments. Of those 62 genes, there was a moderate correlation (Pearson's $r = 0.7$) and a slope of 0.70 with regard to expression differences across these treatments. Interestingly there was no evidence for a difference in the magnitude of expression differences for these 62 genes, using the absolute values of the differences from the contrast (|ORE − SAM| *vs.* |ORE *sd* − SAM *sd*|).

These observations can be contrasted with the effects between treatments (wild type *vs.* sd^{E3}) *within* each background, suggesting the genetic background is having a profound effect on changes in overall expression. Only 5% (120/2290) of genes were shared between comparisons of sd^{E3} and wild type across the two genetic backgrounds. Of the 120 genes that are shared, there is no evidence of correlation in expression levels ($r =$

TABLE 2

Overrepresentation of gene ontology categories suggests that expression differences between genetic backgrounds are largely the result of known developmental regulatory genes that affect specification and determination of Drosophila appendages

Term	Examples of genes in the enriched GO sets
	scalloped^{E3} *vs.* wild type
Tissue death	*Nc, Rab7, br, zip, cbt, cathD, Cp1, Ptpmeg, morgue*
Dorsal/ventral pattern formation	*Spn-A, CSN5, pll, bai, tub, Med, fng, loco, Tehao, tsu, Spn27A, dl, Ser, Khc*
Exocrine system development	*Med, Ptpmeg, eyg, spi, klar, Ser*
RNA localization	*didum, thoc5, thoc6, Hpr1, tsu, chic, ref2, La, Rop, Nxt1, Hel25E, Khc*
Positive regulation of physiological process	*InR, tna, Bgb, br, eIF-4E,Med*
Positive regulation of cellular process	*spi, e(r), iHog, fng, Bgb, br, arm, cyc, tna, eIF-4E, Bka, cbt, dl, lok, lgs*
Catabolism	*dp, Acon, Pgk, Pros54, Smg5*
Cell cycle	*dpa, Myb, fzy, CycD, Cdc27, Cdc37, Cdk9, SMC2, zip, Cks85A, san, Hdac3, HDAC4, Dmn*
	Oregon *vs.* Samarkand
Morphogenesis of an epithelium	*nmo, par-6, cni, l(2)gl, Nmt, Dg, sqd, Rho1, flfl, rin, Moe, nej, cora*
Cell fate commitment	*dlg1, par-1, Dr, ttk, sty, rin, hdc, nej, grh, sina*
Ectoderm development	*sog, shot, gro, Dr, vvl, Dl, ewg, ap, sns, WupA*
Larval or pupal development (*sensu* Insecta)	*ap, bi, hth, sbb, CecB, frc, Dr, l(2)gl, rn, ttk, svp, rin, Moe, nej, how, crol*
Organ morphogenesis	*frc, shot, Dr, rn, grn, Dl, ewg, ap, rin, Moe, nej, how, Tina-1, rg*
Appendage development and morphogenesis	*vg, Dl, nmo, frc, shot, Dr, rn, Rho1, Dl, ap, how, crol*
Regulation of cell differentiation	*Dr, sty, Dl, hdc, sina*
Dorsal/ventral pattern formation	*sog, cni, Dr, sqd, Dl, ap, Tehao*
Tissue death	*CecB, Eig71Ej, Obp99b, l(2)gl, ftz-f1, ap*

0.04). There is also suggestive evidence that the absolute magnitude of expression differences is greater in the ORE background [0.52 *vs.* 0.49, SE 0.018, prob(T) = 0.056, 119 d.f.]. These surprising results suggest that expression differences between sd^{E3} and its wild-type conspecifics differ considerably by background. Thus the majority of differentially expressed genes are not consistent with the second model we proposed, where a common subset of genes mediates the background effect, and their expression differences are proportional to the phenotypic effect of the mutation in each background. These results suggest that any biological significance of gene expression differences must be interpreted with care as differences due simply to genetic background can be as large in magnitude as any specific treatment effect, and it is not immediately clear which changes are functionally relevant.

Evidence that the severity of reduction of gene expression correlates with the severity of the mutant phenotype is ambiguous. Consistent with the idea that the more severe *sd* phenotype should be associated with greater differential expression, a clear majority of genes that differ between mutant and wild-type wing discs are more strongly reduced in expression in the ORE than the SAM background. A total of 1145 of the 1230 probes that are significantly differentially expressed in the mutants showed reduced expression in both backgrounds. Of these, 658 (57.5%) show expression in SAM sd^{E3} that is between that of ORE sd^{E3} and the average of the wild-type backgrounds. However, this means that 487 probes indicate the opposite relationship, namely greater reduction in Samarkand, which is counter to the expectations based on the mutant phenotype.

DISCUSSION

Genetic background is a ubiquitous, though underappreciated, aspect of the genetic architecture of complex traits. For example, the expressivity of individual mutations in the homeotic genes *Ubx* and *Antp* observed across wild-type backgrounds covers the full phenotypic range of allelic series of these genes in a common background (Gibson and van Helden 1997; Gibson *et al.* 1999), and both *Egfr* and *sevenless* effects on photoreceptor determination are more modified by genetic backgrounds than they are by mutations uncovered in second-site modifier screens (Polaczyk *et al.* 1998). Observations such as these have led us to ask whether the genetic architecture of such cryptic variation is similar to that observed for continuous traits and whether it involves segregating alleles that can also contribute to visible variation in a population.

In this study we have examined the effects of two wild-type genetic backgrounds on the expressivity of a loss-of-function allele of *sd*. Our key findings are that (1) a major-effect modifier segregating between our wild-type genetic backgrounds leads to a dramatic reduction of the wing blade in combination with sd^{E3}, (2) the phenotype is mediated through misregulation of a series of developmental patterning genes downstream of *scalloped*, (3) the epistatic interaction between sd^{E3} and

omb^{md} is mediated in a background-dependent manner; and (4) the difference between phenotypes is due to both qualitative and quantitative differences at the level of downstream gene expression.

Interpretation of microarray analysis of gene expression: In the Introduction, we proposed four models for the possible effects of genotype × background interaction on wing disc gene expression. The first model represents a null hypothesis, where differences in phenotype would be mediated primarily by differences in protein concentration or activity that have no visible effect on transcript abundance. This hypothesis is refuted by the observation that >200 genes, many with annotated roles in wing patterning, are differentially expressed in mutant discs of the two genetic backgrounds. Furthermore, *in situ* hybridizations with several of these genes show misregulation at the presumptive wing margin in proportion to the degree of loss of wing blade tissue (Figure 3). A genetic interaction between *sd*^{E3} and one of these targets, *omb*, was demonstrated to enhance the wing phenotype in a background-specific manner (Figure 4), confirming the functional relevance of at least one of the observed changes in transcript abundance.

The second model proposed was that phenotypic expressivity reflects intrinsic differences between the wild-type backgrounds that are amplified by the *sd*^{E3} mutation. If this model is correct we expect to observe that a common subset of genes would be differentially expressed between the two genetic backgrounds, both in the comparison between the wild types (Oregon-R *vs.* Samarkand) and in the comparison between the mutants in each background (Oregon-R *sd*^{E3} *vs.* Samarkand *sd*^{E3}). However, this model is at least partially contradicted by the finding that among the genes that are observed to be differentially expressed between wild-type Samarkand and Oregon-R wing discs, only a small fraction of these distinguish the mutant discs in the two backgrounds (12.6%).

Our data are more generally consistent with the third and fourth models, that the cryptic variation modifies gene expression of a wide range of target genes that do not show differences in normal development. The difference between these models is in the prediction that expressivity involves either differences in degree of modulation of a common set of genes, proportional to the effects of the *sd*^{E3} mutation in each background (model 3), or modulation of different sets of genes in the two backgrounds (model 4). While a number of known Sd-dependent genes (*vg*, *Dll*, *Omb*, and *wg*) are among those consistent with model 3, only 5% of the differentially expressed genes observed were consistent with this model. This suggests that the majority of transcriptional differences that are observed are due to background-specific modulation of genes.

We propose that such differences can be reconciled in the context of developmental cascades. The immediate effect of loss of *sd* transcriptional activity is impaired by its ability to partner with Vestigial to organize development of the wing margin. This has an effect on expression of immediate target genes such as *vg*, *wg*, *Dll*, and *omb*. As a consequence of threshold responses to loss of wing margin specification, some genes farther downstream show complete loss of activation in the Oregon R background, but relatively normal expression in Samarkand (Figure 3). Other genotype-specific responses are also observed, with the result that the snapshot of gene expression profiled in late third-instar wing discs includes expression of hundreds of genes that differ not just from wild type, but also between mutants of the two background classes. Thus, a slight discontinuity that has almost no effect on normal development is amplified into remodeling of as much as a quarter of the wing transcriptome.

As with many genomic studies, with a large number of differentially expressed genes, our results do not explicitly exclude any of the models that we proposed, but instead provide an initial quantitative estimate as to the relative contribution of each of these genetic models to an understanding of genetic background effects. However, the results from these experiments are not conclusive in identifying those changes in expression that modulate the observed differences in the wing morphology between backgrounds for the *sd*^{E3} allele. Indeed, conclusions based solely on the microarray data must be considered provisional given the high error rate and general low repeatability common to such studies. Considerable future work will be required to provide a complete functional dissection of such background effects.

Developing a model for the study of the genetics of background effects: As discussed in the Introduction, there are a number of reasons why the explicit study of genetic background effects should be considered an important avenue of research. As with sensitization screens (Karim *et al.* 1996), mapping of genetic background effects can be used to enrich the list of known genes involved in specific developmental and physiological processes. From the results of this study as well as previously published work (Gibson and van Helden 1997; Polaczyk *et al.* 1998; Atallah *et al.* 2004), it is undeniable that the penetrance and expressivity of a particular mutation are dependent on the genetic background in which they are measured (Nadeau 2001). However, it is unclear whether the genetic background affects the relative ordering of allelic series for specific mutants. Similarly, it is unclear how genetic background may effect epistatic interactions either quantitatively or perhaps even qualitatively.

In this study we demonstrate that the genetic interaction between *sd* and *omb* is entirely background dependent. In the Samarkand background the *sd*^{E3} *omb*^{md} double-mutant combination shows a phenotype that is more severe than either individual mutant

(Figure 4A), while in the Oregon-R background this same combination is qualitatively indistinguishable from the sd^{E3} single mutant (Figure 4B). While this result demonstrates the need to consider the effects of genetic background as a ubiquitous property of genetic systems, it is unclear how generally it will modify epistatic relationships as observed in this study; that is, what proportion of induced genetic modifiers will be background specific? It also begs the question as to the mechanism for this change in epistasis. Do the single- and double-mutant combinations affect gene regulation in a similar manner in one genetic background, but not in the other? Despite conservation of the DNA sequence of genes as well as protein function, genetic interactions need not be conserved between distantly related organisms (Tischler *et al.* 2008). Our results are consistent with the hypothesis that there may be genetic variation for genetic interactions within species (van Swinderen and Greenspan 2005), although this requires further study.

Genetic background effects and cryptic genetic variation: The questions raised here and by others (Felix 2007; Sangster *et al.* 2008) suggest that concurrent with theoretical developments of the potential role of cryptic genetic variation, there is a need to develop suitable experimental model systems to understand the biological basis of genetic background effects. Recent interest has addressed the possible function of cryptic genetic variation with respect to the maintenance of genetic variation and its role during adaptation (Gibson and Dworkin 2004; Masel 2005; Hansen 2006; Barrett and Schluter 2008; Le Rouzic and Carlborg 2008). While considerable attention has been given to the possibility that Hsp90 may act as a capacitor of evolutionary change by hiding the effects of stores of genetic variation that may be exposed by stress (Rutherford and Lindquist 1998; Milton *et al.* 2006; Sangster *et al.* 2007, 2008), there is conflicting evidence that such a process actually modulates genetic variance for quantitative traits (Milton *et al.* 2003, 2005; Debat *et al.* 2006) or that it influences the evolutionary process. Unfortunately, this debate has often obscured the more general finding that results similar to those found for perturbation of Hsp90 activity are observed whenever any visible mutant is introgressed into wild-type backgrounds (Dworkin 2005b; Hall *et al.* 2007). However, to date there has been little effort to discern the identity of the allelic variants that contribute to such background-dependent effects or to understand the functional consequences of these variants. The identification of the allelic variants responsible for such genetic background effects, and examining their potential contribution to variation in natural populations, will be requisite in the advancement of this field.

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