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Morphological evolution caused by many subtle-effect substitutions in regulatory DNA

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Morphology evolves often through changes in developmental genes, but the causal mutations, and their effects, remain largely unknown. The evolution of naked cuticle on larvae of *Drosophila sechellia* resulted from changes in five transcriptional enhancers of *shavenbaby* (*svb*), a transcript of the *ovo* locus that encodes a transcription factor that governs morphogenesis of microtrichiae, hereafter called 'trichomes'. Here we show that the function of one of these enhancers evolved through multiple single-nucleotide substitutions that altered both the timing and level of *svb* expression. The consequences of these nucleotide substitutions on larval morphology were quantified with a novel functional assay. We found that each substitution had a relatively small phenotypic effect, and that many nucleotide changes account for this large morphological difference. In addition, we observed that the substitutions had non-additive effects. These data provide unprecedented resolution of the phenotypic effects of substitutions and show how individual nucleotide changes in a transcriptional enhancer have caused morphological evolution.

The genetic mechanisms underlying morphological evolution remain largely unknown[1,2]. Comparative studies indicate that changes in the timing (heterochrony), location (heterotopy), and level of gene expression have caused much of morphological evolution[3–8]. But, with a few exceptions[9–11], we do not know the specific DNA changes responsible for altered expression, leaving several important questions unanswered. First, it is unclear how many genetic changes underlie new morphologies[12]. Second, we do not know whether multiple substitutions have independent effects or if they contribute instead to epistasis, where the effects of one change are dependent on other changes[13–15]. Third, it has been predicted that the changes that cause morphological evolution have minimal pleiotropic effects[16–18]. Last, we do not know how often transcriptional regulation evolves through deletion and *de novo* creation of enhancers as opposed to subtle modification of existing *cis*-regulatory modules[19–21].

Here we identify the molecular changes in a transcriptional enhancer underlying a case of morphological evolution. To shed light on the interplay between gene expression divergence and morphological evolution, we evaluated the effects of these changes on timing and level of expression and also determined their effects on the resulting phenotype.

Modular enhancers regulate *svb* transcription

Drosophila melanogaster larvae are decorated with a complex pattern of trichomes that results from the differentiation of epidermal cells (Fig. 1a, b). We focus on the dorsolateral epidermis, which differentiates quaternary trichomes in *D. melanogaster* (Fig. 1b) and in most related species[22]. Evolution of *cis*-regulatory regions of the *svb* gene, which encodes a transcription factor that orchestrates trichome morphogenesis[23,24], cause *D. sechellia* larvae to differentiate smooth cuticle, rather than quaternary trichomes[25] (Fig. 1c). This derived phenotype resulted from the specific loss of *svb* expression in quaternary cells (Fig. 1d, e), while *svb* expression is conserved in other epidermal cells, such as those that produce the ventral stout trichomes, called denticles[22].

Through systematic dissection of the ~110-kb *D. melanogaster svb* locus, we identified six embryonic enhancers of ~5 kb[25,26] (Fig. 1f). In *D. sechellia*, five of these six enhancers have evolved reduced activity in quaternary cells[25,26]. One of these enhancers, *E*, drives strong expression in quaternary cells and in the ventral denticle cells of *D. melanogaster* embryos[25]. The orthologous *E* region from *D. sechellia* drives greatly diminished expression in quaternary cells, which directly contributed to trichome pattern evolution[25], while expression driven by this enhancer in ventral cells is conserved[25]. The *E cis*-regulatory element thus represents an attractive target for identifying the individual genetic changes that have contributed to morphological evolution in *D. sechellia*.

We found that the ventral and dorsolateral expression driven by *E* are encoded in two distinct regions—each ~1 kb in length—that are separated by ~1.2 kb (Fig. 1g and Supplementary Fig. 1). The first region, *E3*, drives expression in ventral cells that differentiate denticles (Fig. 1h) and the second region, *E6*, drives mostly dorsolateral expression (Fig. 1i). No smaller constructs from the *E6* region showed equivalent activity; *E6* sub-fragments drove expression that was either strongly reduced, partial, or ectopic (Supplementary Fig. 1). The *D. melanogaster E* region thus comprises two *cis*-regulatory modules: *E3*, which drives expression in ventral cells, and *E6*, the minimal region that can drive a coherent pattern of expression in quaternary cells.

A *svb* enhancer evolved by level and timing changes

To assay the evolutionary modification of *E* activity between *D. melanogaster* and *D. sechellia*, for each species we generated *E10* constructs, which included both the evolving *E6* region and the conserved *E3* region. The *E3* region provided an internal control of conserved expression (Fig. 2e, f). The *D. melanogaster E10* construct (*mel_E10*) drove expression in dorsal cells beginning at stage 12–13 (Fig. 2a, c).

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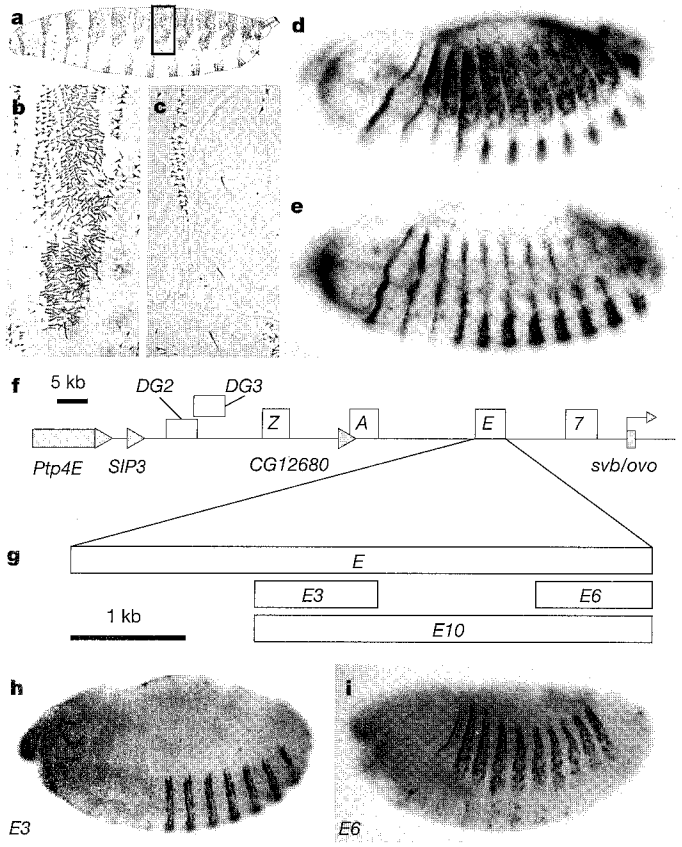

Figure 1 | The pattern of trichomes has evolved between *Drosophila* species owing to changes in the enhancers of the *svb* gene. **a**, Lateral view drawing of a first instar larva of *D. melanogaster*. The rectangle indicates the region shown in **b** and **c**. **b**, **c**, The pattern of dorsolateral trichomes on the fourth abdominal segment of *D. melanogaster* (**b**) and *D. sechellia* (**c**). Some of the dorsolateral cells differentiate thin 'quaternary' trichomes in *D. melanogaster* and naked cuticle in *D. sechellia*. **d**, **e**, Pattern of *svb* RNA expression in stage 14 embryos of *D. melanogaster* (**d**) and *D. sechellia* (**e**). **f**, Diagram illustrating the location of the six enhancers of *svb* (open boxes). The enhancers *7*, *E* and *A* were referred to as *proximal*, *medial* and *distal*, respectively, in ref. 25. Genes in the region are indicated with grey boxes and only the first exon of *svb* is shown. **g**, Summary of the dissection of the *E* enhancer in *D. melanogaster*. Boxes indicate the enhancer constructs discussed in the text. **h**, The *E3* region drives expression in ventral stripes. **i**, The *E6* region drives expression in quaternary cells.

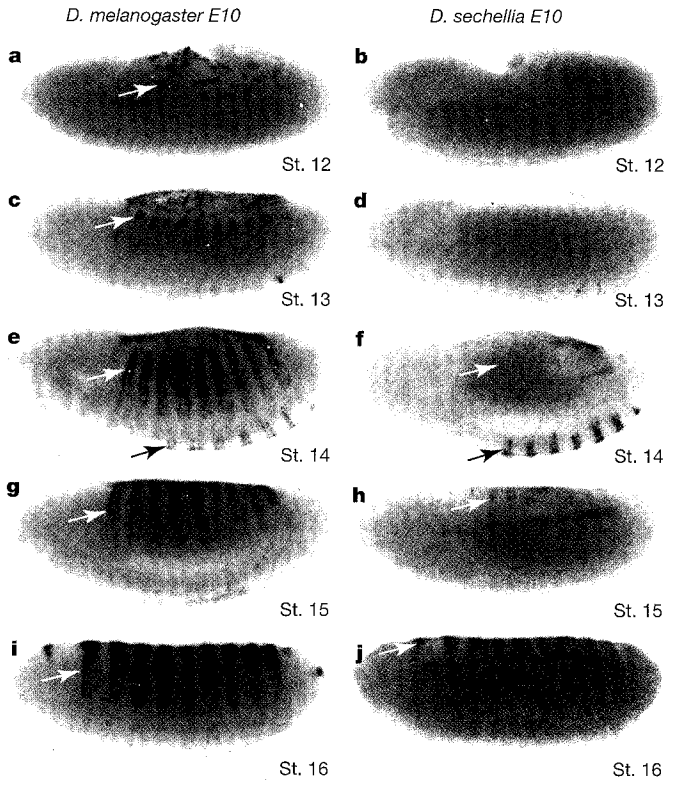

Figure 2 | *D. sechellia E6* shows decreased and delayed expression relative to *D. melanogaster E6*. **a**, **c**, **e**, **g**, **i**, The *D. melanogaster E10* construct drives expression that is detected first in the most dorsal cells of stage (St.) 12 embryos (**a**). This expression strengthens and spreads laterally through stages 13 (**c**), 14 (**e**), 15 (**g**) and 16 (**i**). **b**, **d**, The *D. sechellia E10* construct does not drive detectable expression in stage 12 (**b**) or 13 (**d**) embryos. **f**, **h**, **j**, Dorsal expression (white arrows) is detected in only some stage 14 embryos (**f**) and is clearly observable in stage 15 and 16 embryos (**h**, **j**). **e**, **f**, Both the *D. melanogaster* and *D. sechellia E10* constructs drive similar expression in ventral cells (black arrows).

This pattern strengthened and spread to more lateral cells in later stages (Fig. 2e, g). In stage 16 embryos, *mel_E10* expression persisted in many dorsal and lateral cells (Fig. 2i), while endogenous *svb* messenger RNA is not present at this stage (data not shown). These constructs therefore produce artificially high levels of mRNA in late-stage embryos. This experimental artefact allowed discovery of the surprising fact that, whereas the *D. sechellia E10* (*sec_E10*) does not drive expression before stage 14 (Fig. 2b, d, f), it does drive expression in quaternary cells in late-stage embryos (Fig. 2h, j), albeit at a much lower level than does *mel_E10*. In a separate set of experiments, we confirmed that the *D. sechellia E6* region indeed drives this late dorsal expression (data not shown), indicating that it retains some weak and heterochronic expression. In contrast, the ventral expression driven by *sec_E10* matched the timing and levels driven by *mel_E10*. These data therefore show that conserved ventral expression and divergent dorsal expression of the *E10* regions from *D. melanogaster* and *D. sechellia* is correlated with the patterns of trichomes produced by each species, further localizing evolutionary changes to within the *E6* region.

The *E6* enhancer evolved at an accelerated rate

Next we attempted to identify the DNA changes that caused the evolutionary shift in *E6* function. We compared the sequences of the *E6* region between *D. sechellia* and five closely related species, all of which, like *D. melanogaster*, produce a dense lawn of quaternary trichomes. Multiple sequence alignment allowed us to identify thirteen substitutions and one single-nucleotide deletion that are unique to *D. sechellia* (Fig. 3a, b and Supplementary Fig. 2). These *D. sechellia*-specific substitutions are located in a region of ~ 500 bp (the 'focal region') of otherwise high sequence conservation, even in *D. sechellia* (Fig. 3a).

Given the functional importance of *E6*, we examined whether this apparent clustering of substitutions within a highly conserved block represented an unusual substitution rate. We sequenced the *E6* focal region from eight additional isolates of *D. sechellia*. All nine *D. sechellia* sequences were identical (data not shown), which is consistent with the low levels of polymorphism detected in other regions of the *D. sechellia* genome[27,28]. The absence of polymorphism in the *E6* region in *D. sechellia* prevented us from using common tests of selection that rely on allele frequencies[29]. Instead, we analysed substitution rates in the *D. sechellia* and *D. simulans* lineages, using *D. melanogaster* as an outgroup[30]. We observed a significant increase in *D. sechellia* divergence, compared to *D. simulans*, in the focal region of *E6* (Fig. 3c; Tajima's relative rate test, $\chi^2 = 6.25$, $P = 0.012$, 503 alignable bases). To determine whether this pattern of accelerated divergence reflects simply an accelerated evolutionary rate of substitution at this genomic locus in *D. sechellia*, we sequenced ~9,000 bp of DNA flanking the focal region, which does not include any of the other evolved enhancers, both from *D. sechellia* and from *D. simulans*. The ~9,000 bp region has not evolved at significantly different rates in the two lineages (Fig. 3d; Tajima's relative rate test, $\chi^2 = 0.56$, $P = 0.45$, 7,072 alignable

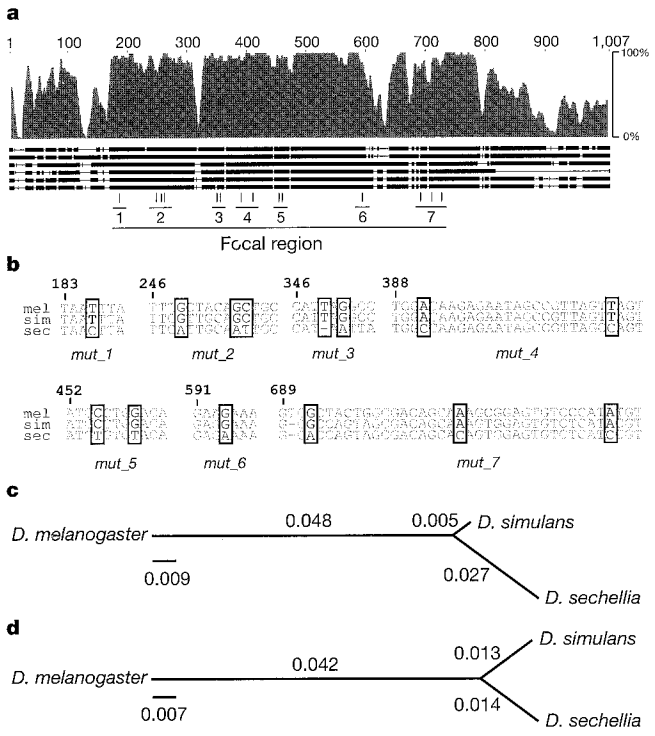

Figure 3 | Sequence conservation of the *E6* region and location of the *D. sechellia*-specific substitutions. **a**, The aligned *E6* sequences from *D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. sechellia*, *D. yakuba* and *D. erecta* are represented as thick horizontal lines, with thin regions indicating gaps in the alignments. (Full alignment is provided in Supplementary Fig. 2.) Sequence conservation over a 10-bp sliding window is represented above by the height of the grey bars. The positions of *D. sechellia*-specific substitutions are indicated with vertical red lines, the seven clusters of substitutions are indicated below the red lines, and the 'focal region' is labelled. **b**, Sequences of the seven regions containing the *D. sechellia*-specific substitutions (enclosed in rectangles) with the aligned sequences from *D. melanogaster* (*mel*), *D. simulans* (*sim*) and *D. sechellia* (*sec*). **c**, **d**, Evolutionary trees of the *E6* focal region (**c**) and 9 kb outside of the focal region (**d**), where branch lengths are proportional to the substitution rate. Numbers indicate number of substitutions per site on average.

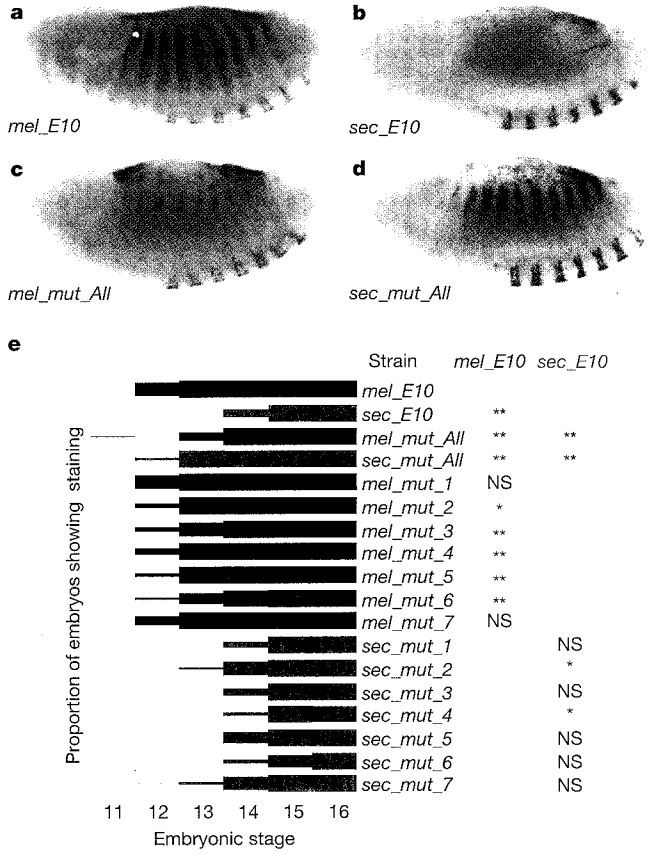

Figure 4 | Evolutionary engineering of the *E10* enhancer reveals the role of evolved substitutions in altering the levels and timing of expression. **a**, **b**, Reporter gene expression driven by the *D. melanogaster E10* (**a**) and *D. sechellia E10* (**b**) constructs in stage 14 *D. melanogaster* embryos. **c**, Introducing all seven clusters of *D. sechellia*-specific substitutions into a *mel_E10* construct (*mel_mut_All*) strongly reduces dorsal expression in stage 14 embryos. **d**, Introducing the respective *D. melanogaster* nucleotides into a *sec_E10* construct (*sec_mut_All*) almost completely restores dorsolateral expression in stage 14 embryos. **e**, The onset of expression driven by the *E10* and *mut* enhancers was quantified by counting the proportion of embryos showing dorsolateral expression at each of six embryonic stages. The *mel_mut_All* and *sec_mut_All* show strong changes in the onset of expression compared with the respective wild-type constructs. Five of the *D. melanogaster mut* lines also show delayed onset of expression compared with the *mel_E10* construct. Two of the *sec_mut* lines show significant differences in the onset of expression compared with *sec_E10*. The *E10* and *mut_All* comparisons were made at stage 13 and the individual cluster *mel_mut* and *sec_mut* comparisons were made at stages 12 and 14, respectively. Sequential Bonferroni test *P* values: $*P < 0.05$, $**P < 0.01$. NS, not significant.

bases). In the *D. sechellia* lineage, the focal region experienced a significantly higher substitution rate (4.8 times higher) than did the flanking regions (Fisher's exact test, two-tailed $P = 0.016$). Therefore, when compared to neighbouring regions, the focal region of *E6* evolved at a faster rate in the *D. sechellia* lineage, indicating that it has evolved under positive selection[31], or relaxed constraints[32], or both.

Substitutions in *E6* altered enhancer function

To assay the effect of the *D. sechellia*-specific substitutions in *E6* on enhancer activity, we introduced all of these substitutions into *mel_E10*. We also performed the reciprocal experiment by reversing the *D. sechellia*-specific substitutions to the *D. melanogaster* sequence in *sec_E10*. To enable trichome rescue experiments, the mutated *E10* versions were placed upstream of a *svb* complementary DNA that contained a heterologous tag in the 3′ untranslated region (UTR), which allowed us to differentiate expression driven by the transgene from expression driven by the endogenous *svb* gene.

In stage 14 embryos, the *D. melanogaster E10* construct carrying all of the *D. sechellia*-specific substitutions (*mel_mut_All*) drove substantially weaker expression in quaternary cells than did *mel_E10* (Fig. 4a, c). Conversely, the *D. sechellia E10* carrying all of the 'reverse' substitutions to the *D. melanogaster* state (*sec_mut_All*) drove substantially stronger dorsal expression than did *sec_E10* (Fig. 4b, d). These manipulated enhancers did not perfectly reproduce the temporal and spatial differences between *mel_E10* and *sec_E10* (Fig. 4), indicating that at least one other substitution in *E10* contributed to the functional divergence of these enhancers. All together, these results confirm that at least one of the *D. sechellia*-specific substitutions in the *E6* region caused most of the species difference in *E6* function.

Many substitutions caused morphological evolution

Next we asked which of the *D. sechellia*-specific substitutions caused the altered function of *E6* in *D. sechellia*. As the *D. sechellia*-specific substitutions in the *E6* enhancer appeared to be clustered in seven regions (Fig. 3a), we mutated separately these seven clusters of nucleotides (Fig. 3b) from the *D. melanogaster* to the *D. sechellia* sequence in *mel_E10*. We also performed the reverse experiment, separately mutating each of seven clusters from the *D. sechellia* to the *D. melanogaster* sequence in *sec_E10*. Some of the *D. melanogaster* constructs with individual mutated clusters showed weaker lateral expression in stage 14 embryos than *mel_E10* did (data not shown). Quantification of the

onset of expression revealed further that five of seven of the *D. melanogaster* mutated enhancers drove significantly delayed expression when compared to *mel_E10* (Fig. 4e and Supplementary Table 1). In the reciprocal experiments, some *sec_E10* constructs with clusters of *D. melanogaster* substitutions drove slightly stronger dorsolateral expression in quaternary cells than did *sec_E10* (data not shown). Some of these *sec_mut* constructs drove a significantly altered onset of expression than did *sec_E10*, but these differences were not of large magnitude (Fig. 4e and Supplementary Table 1). Most importantly, no single cluster of substitutions in either direction recapitulated the temporal onset of expression observed when all substitutions were introduced together (Fig. 4e).

These results indicate that at least five of the *D. sechellia*-specific substitutions in the *E6* region contributed to the functional divergence of this enhancer. We therefore quantified the ability of these constructs to rescue trichomes in an embryo that lacked endogenous *svb* activity (Fig. 5). We tested first whether *mel_E10* and *sec_E10* could rescue the production of trichomes with normal morphology in the correct spatial domains (Fig. 5a–c, i). *mel_E10* rescued many, but not all, of the quaternary trichomes (Fig. 5c, m, n) and recovered many ventral trichomes (Supplementary Fig. 3). The incomplete rescue of both dorsal and ventral trichomes was expected, because multiple *svb* enhancers together contribute to the complete pattern of *svb* expression[26]. *sec_E10* rescued ventral trichomes as well as *mel_E10* did (Supplementary Fig. 3), but recovered only a few dorsal trichomes (Fig. 5i, m), consistent with the conserved and evolved functions of *E10*. Therefore, this rescue assay provides a reliable readout of the normal function of *svb* enhancers.

Because the *D. sechellia*-specific substitutions in *E6* are sufficient to almost completely recapitulate the differences in expression patterns between the species, we asked whether these changes were sufficient to modify trichome patterning. Introduction of all of the *D. sechellia*-specific substitutions from *E6* into *mel_E10*, *mel_mut_All*, caused larvae to produce many fewer trichomes than did *mel_E10*, and thus to look more like *D. sechellia* (Fig. 5d, m, n). Conversely, larvae carrying the reversed substitutions in a *D. sechellia* background (*sec_mut_All*) looked more like *D. melanogaster* larvae (Fig. 5l, m).

To determine how many substitutions cause this species difference in enhancer activity, we tested whether each cluster of substitutions influenced trichome patterns. In *mel_mut_2*, *mel_mut_3*, *mel_mut_4* and *mel_mut_5*, the *D. melanogaster* to *D. sechellia* substitutions reduced the number of trichomes produced by 4.6–33.5% (Fig. 5e–h, m, n and Supplementary Table 3). In contrast, in only *sec_mut_2* and *sec_mut_3* did the *D. sechellia* to *D. melanogaster* substitutions increase the number of trichomes by 9.9–14.6% (Fig. 5j, k, m, n and Supplementary Table 3).

Larvae carrying *mel_mut_All* differentiated significantly more trichomes than did larvae carrying *sec_E10*. The opposite is also true; *sec_mut_All* did not rescue as many trichomes as did *mel_E10*. Thus, additional substitutions within *E10*, other than those we tested, might also have contributed to the morphological difference between *D. melanogaster* and *D. sechellia*.

The functional rescue experiments show that at least four clusters of substitutions in *E6* can alter trichome patterning on their own. Both the onset of expression data and the trichome rescue data indicate that the *D. sechellia*-specific substitutions show epistasis with respect to each other and with respect to the remaining *E10* sequence. Indeed, the magnitude of the effect of mutating all seven clusters of substitutions together on trichome patterning is not recapitulated by summing up the effects of all clusters acting alone (Fig. 5m, n and Supplementary Table 3). The impact of each substitution on larval morphology is thus partly dependent on which other substitutions are already present.

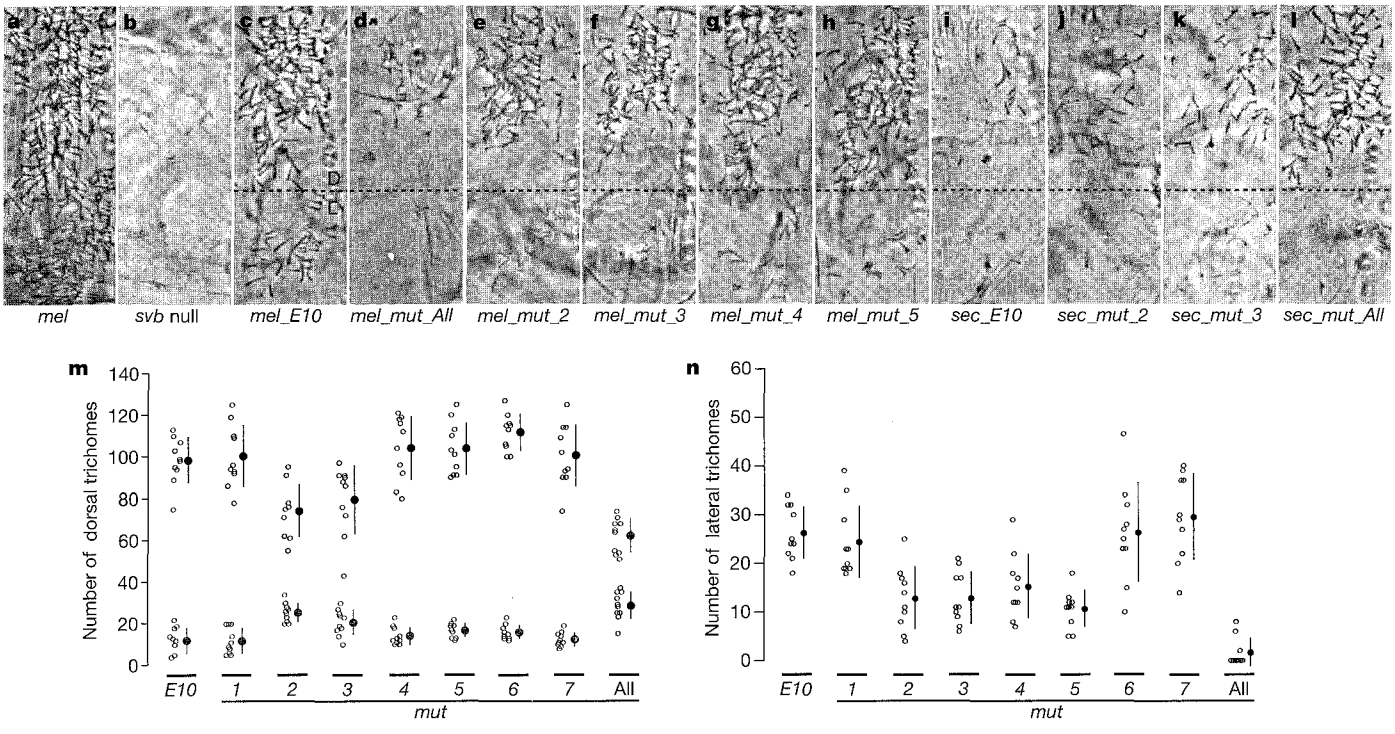

Figure 5 | Effect of the engineered substitutions on trichome rescue in dorsal and lateral regions of the sixth abdominal segment of first instar larvae. **a**, Wild-type *D. melanogaster*. **b**, *svb* null. **c**, *mel_E10* in a *svb* null background. The dorsal (D) and lateral (L) regions where trichomes were counted are delimited with a dashed line. **d–h**, *mel_E10* constructs carrying all *D. sechellia* substitutions (**d**), or cluster 2 (**e**), 3 (**f**), 4 (**g**), or 5 (**h**) substitutions in a *svb* null background. **i**, *mel_E10* in a *svb* null background. **j–l**, *sec_E10* constructs carrying cluster 2 (**e**), 3 (**f**), or all *D. melanogaster* substitutions (**l**). **m**, **n**, Number of trichomes rescued by the *mel* (black) and *sec* (red) constructs in the dorsal (**m**) and lateral (**n**) regions. All larvae carrying *sec_mut* constructs differentiated zero trichomes in the lateral region, and for clarity these data are not shown in **n**. Open circles represent counts for each individual. Closed circles and lines indicate the means and standard deviations, respectively. Grey shading encompasses the constructs with trichome counts that were significantly different from the *E10* construct of the respective species ($P < 0.05$, Dunnet's test).

Note that there is not perfect congruence between the analysis of gene expression patterns and the functional readout of trichome number. For example, *mel_mut_6* altered expression timing, but not trichome number. This suggests that subtle expression differences may not always correctly predict the effects of genetic changes on morphological evolution.

Discussion

We have identified molecular changes in a *cis*-regulatory region that contributed to a morphological difference between closely related species. We found that, taken individually, each genetic change in a transcriptional enhancer had a relatively small effect on gene expression and on the final phenotype, but that when they were combined, they produced a large morphological difference. The substitutions that contributed to morphological evolution showed substantial epistasis, both with respect to the background *E10* construct and with respect to the other substitutions in *E6*. It is impossible to know the actual order in which these substitutions occurred nor whether all of the mutations went to fixation independently or whether some co-segregated. Thus we focused on the effects of individual clusters of substitutions in the background of the parental species.

Our results indicate that at least five substitutions in the *E10* region—at least four in the mutated clusters and at least one other site—contributed to altered function of the *E6* enhancer in *D. sechellia*. Similarly, a study of pigmentation differences among *D. melanogaster* populations showed that multiple polymorphisms of small effect in enhancers of the gene *ebony* account for large phenotypic differences[10]. We propose that enhancer structure influences the patterns of genetic change. When the function of a *cis*-regulatory module relies on multiple transcription-factor-binding sites, each with a small effect on expression, evolution may require changes of a large number of such sites to cause a significant phenotypic change.

Detecting the action of natural selection on specific non-coding genomic regions remains a major challenge for evolutionary genetics[33–35]. The accelerated substitution rate that we observed in the *D. sechellia E6* focal region indicates that this region experienced either positive selection[36], relaxation of purifying selection[37], or both. In addition, none of the *D. melanogaster* to *D. sechellia* mutations led to a significant increase in trichome number, and none of the reciprocal mutations led to a decrease in trichome number. That is, along the lineage leading to *D. sechellia*, the *E6* enhancer seems to have accumulated only substitutions that decrease trichome number. These observations also are consistent with the action of directional selection[36], unless random mutations in this enhancer preferentially cause loss of expression.

When we reverted the *D. sechellia*-specific substitutions to the ancestral state, the *D. sechellia E10* construct regained most of the functionality present in the *D. melanogaster E10* construct. Thus, in principle, descendants of modern *D. sechellia* could re-evolve at least some trichomes through the accumulation of single-nucleotide substitutions in an existing enhancer. Our results contrast with other recent studies of *cis*-regulatory evolution that have discovered large deletions in transcriptional enhancers[9]. For example, the wholesale deletion of an enhancer caused the loss of pelvic structures in some stickleback populations[11]. Although this is a striking result, large deletions may contribute to morphological evolution only rarely. For example, enhancer deletions may have deleterious pleiotropic effects, because many single enhancer 'modules' in fact encode expression in multiple domains[1,38–40]. In addition, new expression patterns may sometimes evolve through modification of existing enhancers[21,41,42]. Widespread deletion of *cis*-regulatory DNA may thus reduce the evolutionary potential of existing enhancers. It is worth noting that the stickleback populations with different pelvic structures diverged less than 10,000 years ago[11]. Our study focuses on morphological differences between species that diverged approximately 500,000 years ago. The markedly different genetic architecture discovered in these two cases may indicate that different kinds of mutations are selected over different evolutionary timescales[2]

Our results suggest an additional explanation for the predominance of single-nucleotide substitutions that have altered *E6* function. Some constructs carrying large deletions of the *E6* element generated ectopic expression (Supplementary Fig. 1). This may be a general feature of enhancers that require multiple activation and repressive activities to define a precise spatiotemporal pattern of expression[43,44]. In such cases, large insertions or deletions may result in ectopic expression and, potentially, in dominant pleiotropic effects. In contrast, single-nucleotide substitutions within activator and repressor binding sites may result in subtle changes in expression with minimal pleiotropic effects. For example, substitutions that lead to heterochrony in enhancer activity can modify a transcriptional program without deleterious effects on development. Such a heterochronic shift in enhancer activity could result from either downregulation of enhancer activity or from a temporal delay in the initiation of enhancer activation. Either or both kinds of events may have occurred in the *D. sechellia* lineage.

METHODS SUMMARY

Embryos were collected and fixed using standard conditions and β-galactosidase expression was detected with immunohistochemistry using a rabbit anti-β-galactosidase antibody (Cappel) used at 1:2,000 and an anti-rabbit antibody coupled to HRP (Santa Cruz Biotech), also used at 1:2,000. Staining was developed with DAB/Nickel.

To detect the expression of transgenic *svb* transcripts, we made an RNA probe complementary to the *lacZ* and *SV40* sequence in the 3′ UTR of the *svb* cDNA using the Dig RNA labelling kit (Roche). We tested for heterochronic changes in the onset of transgene expression by comparing the proportion of embryos showing staining between constructs at a single stage. We then tested for differences in the proportions of stained embryos with the Barnard test using a sequential Bonferroni correction for multiple tests.

For trichome rescue experiments, we cloned *D. melanogaster* and *D. sechellia E10* into pRSQsvb[26]. Mutant plasmids were generated using site-directed mutagenesis (Genescript USA). Constructs were integrated into the attP site of line M(3×P3-RFP.attP)ZH- 86Fb; M(vas-int.Dm)ZH-2A. Males homozygous for the transgene were crossed to *svb*$^{-}$/FM7c;*twi*::*GFP* females. Non-fluorescent first instar larvae from this cross were mounted on a microscope slide in a drop of Hoyer's:lactic acid (1:1). Cleared cuticles were imaged with phase-contrast microscopy. Dorsal and lateral regions were defined using morphological landmarks and programmed as macros in ImageJ software[45]. Trichomes were counted using the cell-counter option of ImageJ. We performed pairwise comparisons of trichome numbers between the wild-type construct and each mutated construct and the statistical significance of comparisons was determined with Dunnet's test. Full details of methods used can be found in Supplementary Information.

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