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Regulatory evolution of *shavenbaby/ovo* underlies multiple cases of morphological parallelism

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Cases of convergent evolution that involve changes in the same developmental pathway, called parallelism, provide evidence that a limited number of developmental changes are available to evolve a particular phenotype[1]. To our knowledge, in no case are the genetic changes underlying morphological convergence understood. However, morphological convergence is not generally assumed to imply developmental parallelism[2]. Here we investigate a case of convergence of larval morphology in insects and show that the loss of particular trichomes, observed in one species of the *Drosophila melanogaster* species group, has independently evolved multiple times in the distantly related *D. virilis* species group[3]. We present genetic and gene expression data showing that regulatory changes of the *shavenbaby/ovo* (*svb/ovo*) gene underlie all independent cases of this morphological convergence. Our results indicate that some developmental regulators might preferentially accumulate evolutionary changes and that morphological parallelism might therefore be more common than previously appreciated.

The pattern of microtrichiae (hereafter referred to as trichomes) on the ventral surface of first-instar larvae seems to be conserved across the genus *Drosophila*, whereas the dorsal and lateral surfaces have repeatedly evolved different patterns[3]. In most species, three kinds of trichome are produced in a specific pattern on the dorsal surface[4]. In a single species of the *D. melanogaster* subgroup, *D. sechellia*, many thin trichomes have been lost and the cells instead differentiate naked cuticle[3,5].

The patterning of dorsal trichomes is an interesting case of convergent morphological evolution, because four species from the *D. virilis* species group (*D. ezoana*, *D. borealis* eastern, *D. lacicola* and *D. montana*) also show evolutionary loss of the thin trichomes[3] (Supplementary Fig. 1). All other species of the *D. virilis* species group that we have examined (*D. americana*, *D. borealis* western, *D. canadiana*, *D. flavomontana*, *D. kanekoi*, *D. littoralis*, *D. lummei*, *D. novamexicana* and *D. virilis*) and the more distantly related *D. arizonae* possess a lawn of trichomes similar to that observed in *D. melanogaster*. By mapping these phenotypes onto a recent molecular phylogeny of the *D. virilis* species group[6], we can infer that at least three evolutionary transitions are required to explain the current distribution of trichome loss (Fig. 1). The convergence of trichome patterns in different fly lineages indicates that these changes might be driven by natural selection[7,8], although the selection pressure has not yet been identified. In addition, the evolutionary loss of trichomes in first-instar larvae mirrors an ontogenetic loss of the same trichomes in second-instar and third-instar larvae[9] in all species we have examined, suggesting that these trichomes have a special function in first-instar larvae.

In theory, many genes might have evolved to alter the patterning of larval trichomes. For example, the *wingless* (*wg*) and *hedgehog* (*hh*) pathways and the *lines* gene are involved in patterning the trichomes on the dorsal epidermis[4,10]. It might therefore be interesting to test whether evolution of patterning genes involved in segmentation can account for the evolution of trichome patterns. However, it has been shown[3] that six genes involved in segmentation (*wg*, *gooseberry-distal*, *patched*, *engrailed*, *abdominal-A* and *hunchback*) are expressed identically in 12 species of the *D. virilis* species group, indicating that differences in trichome patterning might have evolved by changes in genes downstream of the segmentation pathway.

We have previously reported, after a full-genome genetic scan, that regulatory evolution at the *svb* gene accounts fully for the difference in trichome pattern between *D. sechellia* and other species of the *D. melanogaster* species group[5]. It has been shown[11] that the transcription factor Svb acts to switch cells between naked cuticle and the production of trichomes. In *D. melanogaster* embryos, *svb* is genetically required for trichome formation; when *svb* is expressed in a cell, that cell autonomously differentiates trichomes, whose morphology is determined by other patterning genes[4,10]. Therefore *svb* integrates numerous sources of information (including the *wg*, *hh*, DER (for *Drosophila* epidermal growth factor receptor), homeotic and dorsal–ventral patterning systems) to specify the final pattern of trichomes.

To determine the genetic nature of the phenotypic differences observed in the *D. virilis* species group, we performed interspecific

genetic crosses. Although most *D. virilis* group species do not interbreed[12], we succeeded in obtaining interspecific hybrid larvae in three cases. First, we crossed *D. borealis* eastern females with *D. montana* males, both of which have a naked phenotype (Fig. 1 and 2a, b). Four larvae of unknown sex were recovered that all had the naked phenotype (Fig. 2c). This cross suggests that the naked phenotype is not caused by different autosomal recessive genes in each species. However, there are several possible genetic explanations for this result: the naked phenotype might be caused by a recessive X-linked gene (if all the larvae were male), by the same recessive X-linked gene in both species (if any of the larvae were female), by the same recessive autosomal gene in both species, or by a dominant allele in one or both of the species.

We succeeded in performing two further crosses that narrowed down these possibilities. We obtained larvae from a cross between *D. virilis* females (Fig. 2d) and *D. borealis* eastern males (Fig. 2a). The anterior halves of these larvae were mounted to analyse the trichome pattern, and DNA was prepared from the posterior half for a polymerase chain reaction assay. Between *D. virilis* and *D. borealis* eastern, we identified a restriction-site polymorphism in the *svb* gene, which is X-linked in *D. melanogaster* and *D. virilis*. Our DNA analysis therefore allowed the determination of both larval sex and the origin of the *svb* allele(s). Two larvae carried only the *D. virilis svb* allele (males); two other larvae carried *svb* alleles from both species (females). All four larvae had a *D. virilis*-like trichome pattern (Fig. 2e), indicating that the *D. virilis* trichome pattern is dominant to the more naked *D. borealis* eastern pattern, just as it is in the *D. melanogaster* species group[5]. In a reciprocal cross between *D. borealis* eastern females and *D. virilis* males, two larvae were obtained. Both carried only the *D. borealis svb* allele (males) and had the *D. borealis* eastern pattern of trichomes (Fig. 2f), confirming the X-linkage of the factor(s) responsible for the difference in trichome pattern. These species carry multiple X-chromosome inversions[13] that are likely to be responsible for the observed absence of recombination between heterospecific X chromosomes (A. Hoikkala, personal communication), which prevented further genetic mapping of the factor(s) involved. Together, the interspecific genetic data show that the presence of trichomes is dominant to the absence of trichomes and that trichome pattern segregates with the X chromosome, which excludes about 80% of the genome but includes the *svb* gene.

Given the central role of *svb* in patterning trichomes in

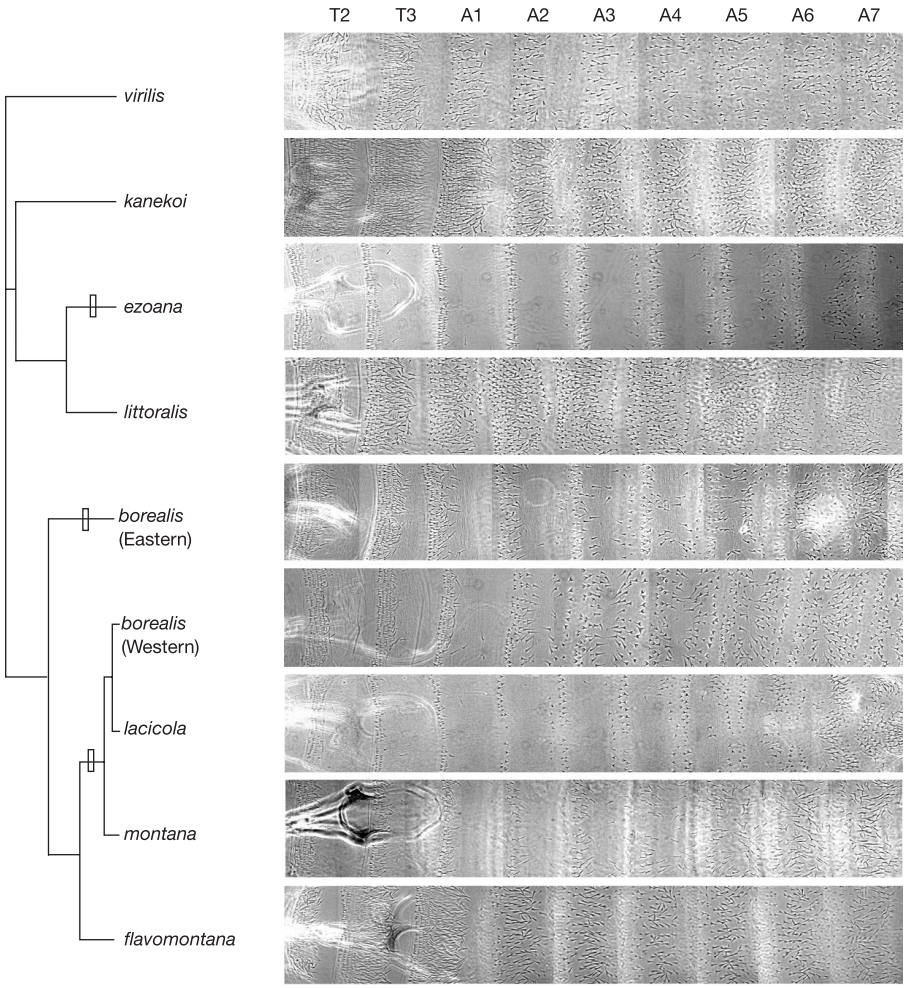

Figure 1 The pattern of dorsal trichomes has evolved repeatedly in the *D. virilis* species group. A phylogeny of the species (adapted from ref. 6) is shown to the left, with branch lengths proportional to molecular divergence. The oldest divergence event is estimated at about 11 Myr BP (ref. 6). The dorsal cuticle is shown for each species, with anterior to the left and the thoracic (T2 and T3) and abdominal (A1–A7) segments labelled at the top. Some species (e.g. *D. kanekoi*, *D. littoralis* and *D. flavomontana*) have an extensive lawn of fine trichomes in every segment, whereas others (*D. borealis* eastern and western, *D. lacicola* and *D. montana*) have naked cuticle to different extents in more anterior segments. *D. ezoana* has the most extreme loss of fine trichomes in this species group. At least three evolutionary transitions are required to explain the distribution of naked and intermediate-naked phenotypes. One parsimonious mapping involves three evolutionary losses of trichomes, assuming that the presence of trichomes is ancestral, indicated by three open boxes on the branches of the phylogeny.

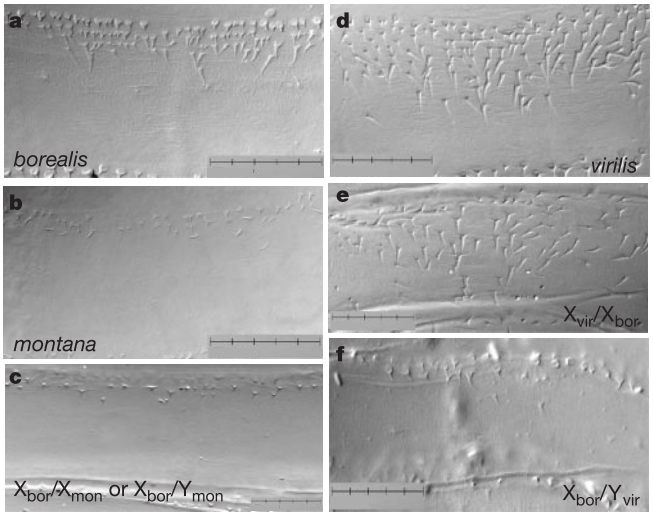

Figure 2 Dorsal first-abdominal segments of parental and species-hybrid larvae. **a**, **b**, *D. borealis* eastern (**a**) and *D. montana* (**b**) show a loss of the fine trichomes. **c**, A hybrid between a *D. borealis* eastern female and a *D. montana* male has the naked cuticle phenotype. The symbols indicate that these larvae carry either an X chromosome from each species (X_{bor}/X_{mon}) or an X chromosome from only *D. borealis* eastern (X_{bor}/Y_{mon}). **d**, *D. virilis* has fine trichomes over much of this segment. **e**, A hybrid between a female *D. virilis* and a male *D. borealis* eastern has the *D. virilis* pattern of trichomes. Because this larva carries an X chromosome from each species (X_{vir}/X_{bor}), this indicates that the *D. virilis* pattern is dominant to the *D. borealis* eastern pattern. **f**, A hybrid male between a female *D. borealis* eastern and a *D. virilis* male has the *D. borealis* eastern pattern of naked cuticle. This larva carries an X chromosome only from *D. borealis* eastern (X_{bor}/Y_{vir}), indicating the X-linked nature of the gene(s) causing the *D. borealis* eastern pattern. Scale bars, 10 μm divisions.

D. melanogaster and its genetic co-segregation with trichome pattern in the *D. virilis* species group, we tested whether the evolution of divergent trichome patterns could be explained by the evolution of the *svb* expression pattern in embryos from nine species from the *D. virilis* species group. We found that the embryonic pattern of *svb* expression closely matches the pattern of trichomes on the first-instar larval cuticle (Fig. 3). In species with an evolutionary loss of dorsal trichomes, *svb* transcription is absent from corresponding cells that differentiate naked cuticle. This suggests a simple model in which the transcriptional enhancer promoting *svb* expression in the domain that produces thin trichomes is turned off in some lineages.

When analysed in detail, however, the patterning of trichomes indicates a more complicated history of *svb* regulatory evolution. In *D. sechellia* and *D. ezoana*, essentially all thin trichomes are lost from all except the most posterior segments. In contrast, in the *D. montana* subphylad, species fall along a continuum from very hairy (*D. flavomontana*) to almost completely naked (*D. lacicola*), with *D. borealis* eastern and *D. montana* having an intermediate naked phenotype (see Fig. 1). In the intermediate phenotypes, thin trichomes are absent to different degrees from the more anterior segments, whereas they are retained in the more posterior segments. These phenotypic differences might have been thought to imply that different genetic mechanisms were responsible for morphological evolution. However, we again observe a strict correlation between *svb* expression and the trichome patterns along the anterior–posterior axis (Fig. 4), indicating that regulatory changes in *svb* expression are involved in all of these evolutionary events. This might be due either to different changes in the *cis*-regulatory region of *svb* or potentially to different *trans*-acting changes in the different lineages. The latter possibility would imply the evolution of additional genes on the X chromosome controlling *svb* expression. This was genetically proved not to occur between *D. melanogaster* and *D. sechellia*[5]. However, rejecting this hypothesis for the *D. virilis* species group will require the identification and characterization of the appropriate regulatory regions of *svb* from these species.

We found that *svb* regulatory evolution underlies all observed cases of convergent evolution of the larval trichome pattern. In the epidermis, *svb* works like a morphogenetic switch[11], making it an effective point in the developmental cascade to generate alternative trichome patterns. Alterations of broader-acting patterning genes might cause pleiotropic consequences, whereas changes to indi-

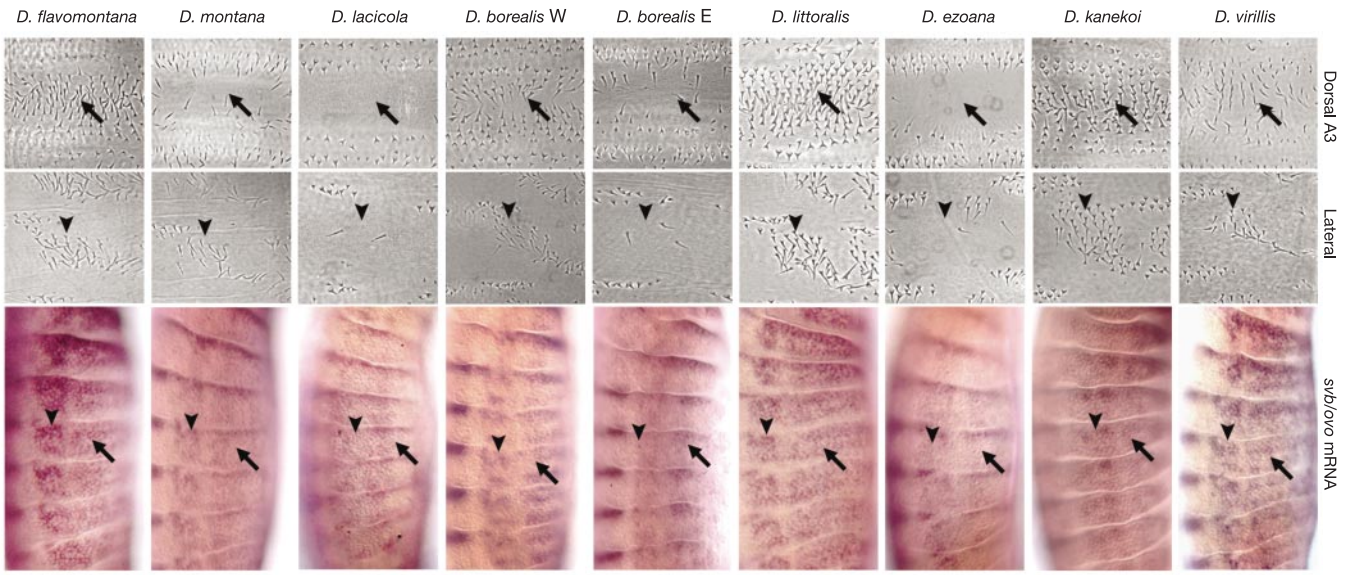

Figure 3 The expression pattern of *svb* is strictly correlated with the pattern of fine trichomes across the *D. virilis* group of species. The dorsal and lateral aspects of the third abdominal segment of first-instar larvae are shown in the first two columns. The embryonic pattern of *svb* expression, as revealed by *in situ* hybridization with a *D. virilis svb* probe, is shown in the right-hand column. The regions of the dorsal and lateral fine hairs are indicated with arrows and arrowheads, respectively, in all panels. The hairs in the lateral panels for *D. borealis* eastern and *D. lacicola* are innervated bristles whose production is not dependent on *svb* function.

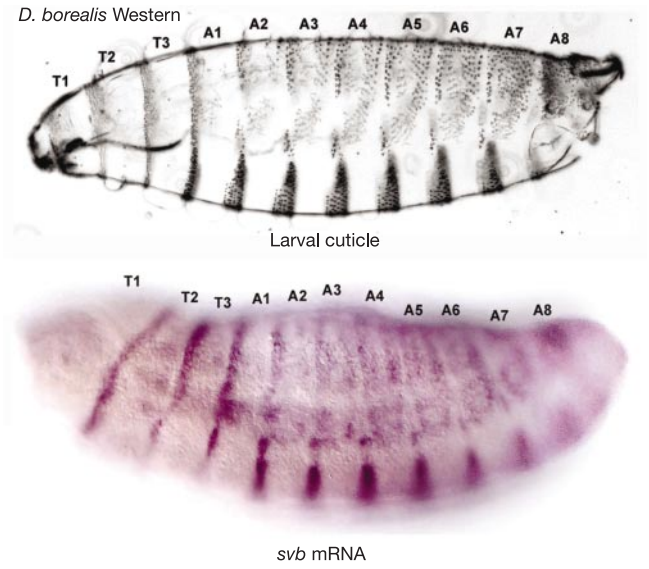

Figure 4 In *Drosophila borealis* western, which has an intermediate dorsal pattern of fine trichomes and naked cuticle, the expression of *svb* (lower panel) is strictly correlated with the pattern of trichomes (upper panel) along the anterior–posterior axis. The thoracic and abdominal segments are labelled.

vidual genes downstream of *svb*, presumably the cytoskeletal genes sculpting the apical extensions that form trichomes, might have no effect. In addition, the modular nature of *svb* regulatory regions permits alterations in part of the cuticular pattern without pleiotropic consequences[5].

It has previously been recognized that organs that are identical by descent—homologous—need not be constructed by identical genetic mechanisms[14]. Our results indicate that changes in the same genetic mechanisms might result in morphological convergence[15–17]. Therefore, observations of identical genetic mechanisms underlying similar morphologies do not necessarily imply homology: phylogenetic information must also be considered. □

Methods

Fly stocks

Flies were obtained from the Species Stock Center (stockcenter.ark.arizona.edu) and maintained on standard cornmeal agar medium: *D. americana* 15010-0951.1, *D. borealis* eastern 15010-0961.0, *D. borealis* western 15010-0961.3, *D. ezoana* 15010-0971.0, *D. flavomontana* 15010-0981.0, *D. kanekoi* 15010-1061.0, *D. lacicola* 15010-0991.0, *D. littoralis* 15010-1001.0, *D. lummei* 15010-1011.1, *D. montana* 15010-1021.6, *D. novamexicana* 15010-1031.0 and *D. virilis* 15010-1051.83. Larval cuticles were prepared with standard protocols and photographed using phase-contrast and differential interference contrast microscopy on a Zeiss Axioplan.

Cloning of *svb/ovo* fragment

A fragment of the *svb/ovo* gene was cloned from *D. virilis* and *D. borealis* by polymerase chain reaction with primers for the conserved zinc-finger region: forward primer, 5′-CGGCCACGGCATCAARAAYCCNYT-3′; reverse primer, 5′-GACACTGTGCACCTTCTGGCAA-3′. We found an *Eco* RV restriction site difference between the *D. borealis* eastern and *D. virilis* alleles, which we used to identify the alleles present in hybrid larvae.

In situ hybridization

Plasmids containing a fragment of the *svb* gene from *D. virilis* were cut with appropriate restriction enzymes and used as templates for the preparation of digoxigenin-labelled sense and antisense RNA using Ambion kits. *In situ* hybridization was performed with standard procedures[11] on 0–24-h embryos collected at 22 °C and fixed with 37% formaldehyde.

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Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain

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Modern pain-control theory[1] predicts that a loss of inhibition (disinhibition) in the dorsal horn of the spinal cord is a crucial substrate for chronic pain syndromes[2]. However, the nature of the mechanisms that underlie such disinhibition has remained controversial[3–6]. Here we present evidence for a novel mechanism of disinhibition following peripheral nerve injury. It involves a trans-synaptic reduction in the expression of the potassium–chloride exporter KCC2, and the consequent disruption of anion homeostasis in neurons of lamina I of the superficial dorsal horn, one of the main spinal nociceptive output pathways[7]. In our