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LETTERS

Morphological evolution through multiple *cis*-regulatory mutations at a single gene

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One central, and yet unsolved, question in evolutionary biology is the relationship between the genetic variants segregating within species and the causes of morphological differences between species. The classic neo-darwinian view postulates that species differences result from the accumulation of small-effect changes at multiple loci. However, many examples support the possible role of larger abrupt changes in the expression of developmental genes in morphological evolution[1–3]. Although this evidence might be considered a challenge to a neo-darwinian micromutationist view of evolution, there are currently few examples of the actual genes causing morphological differences between species[4–10]. Here we examine the genetic basis of a trichome pattern difference between *Drosophila* species, previously shown to result from the evolution of a single gene, *shavenbaby* (*svb*), probably through *cis*-regulatory changes[6]. We first identified three distinct *svb* enhancers from *D. melanogaster* driving reporter gene expression in partly overlapping patterns that together recapitulate endogenous *svb* expression. All three homologous enhancers from *D. sechellia* drive expression in modified patterns, in a direction consistent with the evolved *svb* expression pattern. To test the influence of these enhancers on the actual phenotypic difference, we conducted interspecific genetic mapping at a resolution sufficient to recover multiple intragenic recombinants. This functional analysis revealed that independent genetic regions upstream of *svb* that overlap the three identified enhancers are collectively required to generate the *D. sechellia* trichome pattern. Our results demonstrate that the accumulation of multiple small-effect changes at a single locus underlies the evolution of a morphological difference between species. These data support the view that alleles of large effect that distinguish species may sometimes reflect the accumulation of multiple mutations of small effect at select genes.

Differences in larval trichome pattern between *Drosophila* species offer an attractive model of morphological evolution. Over the past 30 years, numerous studies have identified upstream patterning[11,12] and downstream effector[13] genes regulating trichome development in *D. melanogaster*. Questions about the evolution of trichome patterns can therefore be formulated explicitly within a developmental framework.

Although the pattern of ventral trichomes has been conserved for more than 60 Myr, new dorsal trichome patterns have evolved repeatedly[6,14,15]. In most species of the *D. melanogaster* subgroup, the dorsal and lateral surface displays stout trichomes on 1° and 3° cells and naked 2° cells, and a lawn of fine trichomes on 4° cells[6] (Fig. 1). *D. sechellia* has evolved a trichome pattern in which 4° trichomes were replaced by naked cuticle (Fig. 1h, l)[6]. Interspecific whole-genome genetic mapping demonstrated that the *D. sechellia* 'naked' phenotype is recessive to the 'hairy' phenotype of other species and mapped this evolutionary change to a single X-linked gene, *shavenbaby/ovo* (*svb*)[6]. *Svb* is required cell-autonomously for trichome formation[12] and encodes a transcription factor regulating several classes of effector genes, which collectively build trichomes[13].

In *D. melanogaster*, *D. simulans* and *D. mauritiana*, *svb* is expressed in 1° and 3° dorsal cells and 4° dorsal and lateral cells[16] (Fig. 1). In *D. sechellia*, *svb* is expressed in 1° and 3° dorsal cells but not in the 4° cells (Fig. 1b, d, f), where trichomes are absent[6]. Together with previous genetic analyses, these expression patterns suggest that changes in the *cis*-regulatory region of *svb* underlie this evolved morphological pattern[6].

We therefore sought to identify enhancers that drive *svb* expression. We made a systematic series of *D. melanogaster* reporter constructs, from 50 kilobases (kb) upstream to 20 kb downstream of the first exon of *svb* (Fig. 2a, Supplementary Fig. 1 and Supplementary Table 1). We precisely mapped reporter expression by double-staining for Miniature, the product of a cell-autonomous target of *svb*[13] that accumulates in trichomes (Fig. 2b–g and Supplementary Fig. 2).

We found that three genomic regions drove expression in the epidermis, just before trichome differentiation. Each element contributes to both evolutionarily conserved and evolutionarily derived expression patterns. Dorsal expression of the 'proximal' enhancer started in stage 13 embryos, in 1° and 3° cells (Fig. 2d, and Supplementary Figs 3c, f and 4c, g, k). Beginning at stage 15, expression was observed in some dorsal, but not dorsolateral, 4° cells (Supplementary Figs 3f and 4g). The 'medial' enhancer drove expression in the dorsal 4° cells at stage 13 and later expanded into dorsolateral 4° cells (Fig. 2c, and Supplementary Figs 3b, e and 4b, f, j). The 'distal' enhancer drove expression in thoracic dorsal stripes and lateral 4° cells, starting at stage 14 and strengthening later (Fig. 2b, and Supplementary Figs 3a, d and 4a, e, i). In ventral trichome-producing cells, the proximal and medial enhancers drove strong expression and the distal enhancer drove weak expression (Supplementary Figs 3a–f and 5a–k). The epidermal expression of *svb* therefore seems to be regulated in a complex manner by three separable *cis*-regulatory elements spread over 50 kb.

To determine whether these enhancers have evolved in *D. sechellia*, we identified orthologous *D. sechellia* regions (which differ by 3–5% from the *D. melanogaster* sequences) and assayed their activity as transgenes in *D. melanogaster* (Supplementary Table 1). The *D. sechellia* 'proximal' enhancer drove expression in 1° and 3° dorsal cells in a pattern similar to that of the *D. melanogaster* 'proximal' enhancer (Fig. 2g, and Supplementary Figs 3i, l and 4n, r, v). However, unlike the *D. melanogaster* enhancer, expression from the

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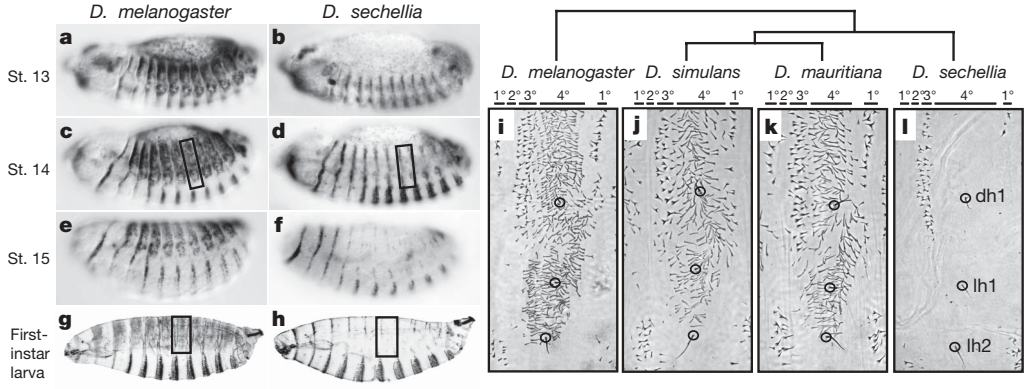

Figure 1 | Modified *svb* expression underlies the evolved trichome pattern of *D. sechellia*. **a**–**f**, *In situ* hybridization of *svb* transcript in stage (st.) 13, 14 and 15 *D. melanogaster* (**a**, **c**, **e**) and *D. sechellia* (**b**, **d**, **f**) embryos (dorsal up; anterior to the left). **g**, **h**, Trichome patterns on first-instar larvae of *D. melanogaster* (**g**) and *D. sechellia* (**h**). Black rectangles identify similar locations on embryos (**c**, **d**) and cuticles (**g**, **h**). **i**–**l**, Cuticle pattern of *D. melanogaster* (**i**), *D. simulans* (**j**), *D. mauritiana* (**k**) and *D. sechellia* (**l**). A phylogeny of the four species[27] is shown above; the clade is 2–3 Myr old[28]. All closely related outgroup species have a *D. melanogaster*-like trichome pattern (D.L.S., unpublished observations), indicating that the *D. sechellia* trichome pattern is derived. Positions of 1°, 2°, 3° and 4° cell types[11] are indicated, and the dorsal hair 1 (dh1), lateral hair 1 (lh1) and lateral hair 2 (lh2) are circled in black.

D. sechellia proximal enhancer was never observed in dorsal 4° cells. Expression of the *D. sechellia* 'medial' enhancer was restricted to dorsal 4° cells (Fig. 2f, and Supplementary Figs 3h, k and 4m, q, u). In contrast with the *D. melanogaster* medial enhancer, expression of the *D. sechellia* enhancer started later and did not extend to the lateral region. The *D. sechellia* 'distal' enhancer drove expression in thoracic stripes, in a similar manner to the *D. melanogaster* enhancer, but expression was observed only in restricted lateral spots (Fig. 2e, and Supplementary Figs 3g, j and 4l, p, t). At the time of trichome formation, each *D. sechellia* enhancer drove a ventral expression pattern similar to that of its *D. melanogaster* counterpart (compare Supplementary Fig. 5i–k with Supplementary Fig. 5t–v).

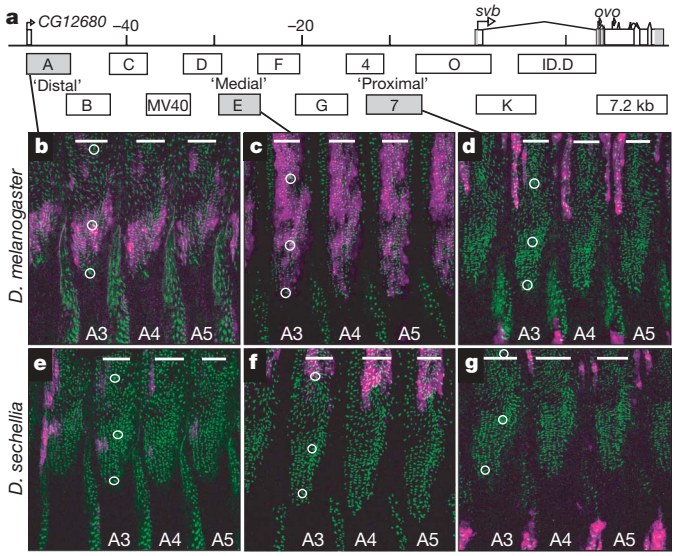

Figure 2 | Three enhancer regions, which collectively recapitulate the *svb* expression pattern, have evolved in *D. sechellia*. **a**, Genomic organization of the *ovo*/*svb* gene and regulatory regions up to *CG12680*, a gene located 50 kb upstream of the first *svb* exon. A systematic series of enhancer constructs, illustrated below the map, was generated (Supplementary Table 1) and only regions labelled as distal (A), medial (E) and proximal (7) drove epidermal expression at the time of trichome formation. **b**–**g**, Reporter patterns of enhancer constructs (purple) from *D. melanogaster* (**b**–**d**) and *D. sechellia* (**e**–**g**) were revealed by double staining of stage-15 embryos with anti-Min to stain trichomes (green) (Supplementary Fig. 2). Images show a lateral view of A3–A5 segments (bars); white circles indicate dh1, lh1 and lh2 sensory bristles. The *Dm*_distal construct (**b**) drives expression in lateral patches of 4° trichomes, *Dm*_medial (**c**) is strongly expressed in dorsal and dorsolateral 4° cells, and *Dm*_proximal (**d**) first drives expression in dorsal 1° and 3° cells and later in the dorsal-most 4° cells (Supplementary Figs 3 and 4). Although still expressed in the thorax, the *Ds*_distal enhancer (**e**) is strongly reduced in lateral 4° cells in the abdomen. At the time of trichome formation, the *Ds*_medial enhancer (**f**) is restricted to the dorsal-most 4° cells. The *Ds*_proximal enhancer (**g**) drives expression in dorsal 1° and 3° cells but was never observed at later stages in 4° cells (Supplementary Figs 3 and 4).

To test the actual function of these enhancers within their native genomic locations for patterning trichomes, we performed high-resolution interspecific recombination mapping[17]. We designed a two-step screen to maximize the probability of identifying recombinants within the *svb* gene. We first screened for recombinants between visible markers that flanked *svb* by about 1.2×10^6 base pairs (bp)[18] and then scored these selected individuals with molecular markers to identify 50 individuals with recombination breakpoints within the *svb* locus (Fig. 3, and Supplementary Information). This experiment provided a resolution of about one recombination breakpoint every 2 kb.

Recombinants that included the entire region upstream of the first *svb* exon from *D. mauritiana* produced trichome patterns indistinguishable from those of *D. mauritiana* (Fig. 3a). Conversely, chromosomes with the upstream *svb* region from *D. sechellia* produced a *D. sechellia*-like trichome pattern (Fig. 3e). These results demonstrate that the change(s) responsible for evolution of the *D. sechellia* phenotype are restricted to the genomic region that contains the three identified enhancers.

If the *D. sechellia* trichome pattern resulted from the evolution of a single site, then only *sechellia*-like and *mauritiana*-like phenotypes would have been observed. Instead, we identified three additional phenotypic classes. First, recombinants that included only the proximal enhancer from *D. mauritiana* produced a few dorsal 4° trichomes (intermediate type 1, Fig. 3b). Second, a chromosome including the medial and proximal enhancers from *D. mauritiana* produced a dense pattern of 4° trichomes in the dorsal and dorsolateral region (intermediate type 2, Fig. 3c). Last, chromosomes that

included only the distal enhancer from *D. mauritiana* produced a patch of dorsolateral and a few dorsal 4° trichomes (intermediate type 3; Fig. 3d). Backcrossing of all viable recombinant lines further ruled out any detectable influence of genomic regions outside *svb* on trichome patterns (Supplementary Table 3).

These genetic results prove that at least three separate changes have evolved in the *svb* upstream region to cause trichome loss in *D. sechellia*. Furthermore, the recombination breakpoints localize functionally evolved sites to genomic positions containing enhancers defined by reporter constructs. The distal *svb* enhancer element includes *CG12680*, which has the potential to encode a short peptide. However, this gene is unlikely to contribute to the evolved difference because *CG12680* is not expressed in embryos (data not shown) and complementation assays implicate *svb* alone as the causal determinant[6]. Finally, the recombinant intermediate phenotypes are similar to the expression patterns of the three individual enhancers (Supplementary Fig. 6). Our combined results therefore imply that each enhancer contains at least one genetic change. These changes may have occurred sequentially by loss of expression from the distal, medial and proximal enhancers, or in any other order (Fig. 4).

Given that laboratory-induced mutations in dozens of genes alter trichome patterns[11,12,19–22], it is striking that multiple mutations at a single locus account for the entire evolved difference. *Svb* seems peculiar in the network of genetic interactions that establish the trichome pattern, because it sits at the nexus of the upstream patterning genes and the downstream effector genes[13,23]. Although trichome pattern could be changed by altering any of several upstream genes, these changes would probably produce pleiotropic effects on other developmental processes. In contrast, none of the known downstream genes is sufficient on its own to prevent or promote trichome formation. Thus, changes at *svb* enhancers may provide the only available genetic mechanism to evolve trichome patterns without pleiotropic consequences.

Our results provide experimental evidence that the conflicting views of micromutationism and macromutationism can actually reflect observations of the same molecular mechanisms at different levels of resolution. Specifically, genes at integrative positions in developmental networks may be genetic 'hotspots' for evolutionary changes that differentiate species, although the individual mutations contributing to this change may be of smaller effect. Although results recently obtained from a broad range of species are consistent with this interpretation[4,24–26], only additional fine-scale functional analyses of morphological differences between species will allow a robust test of this hypothesis.

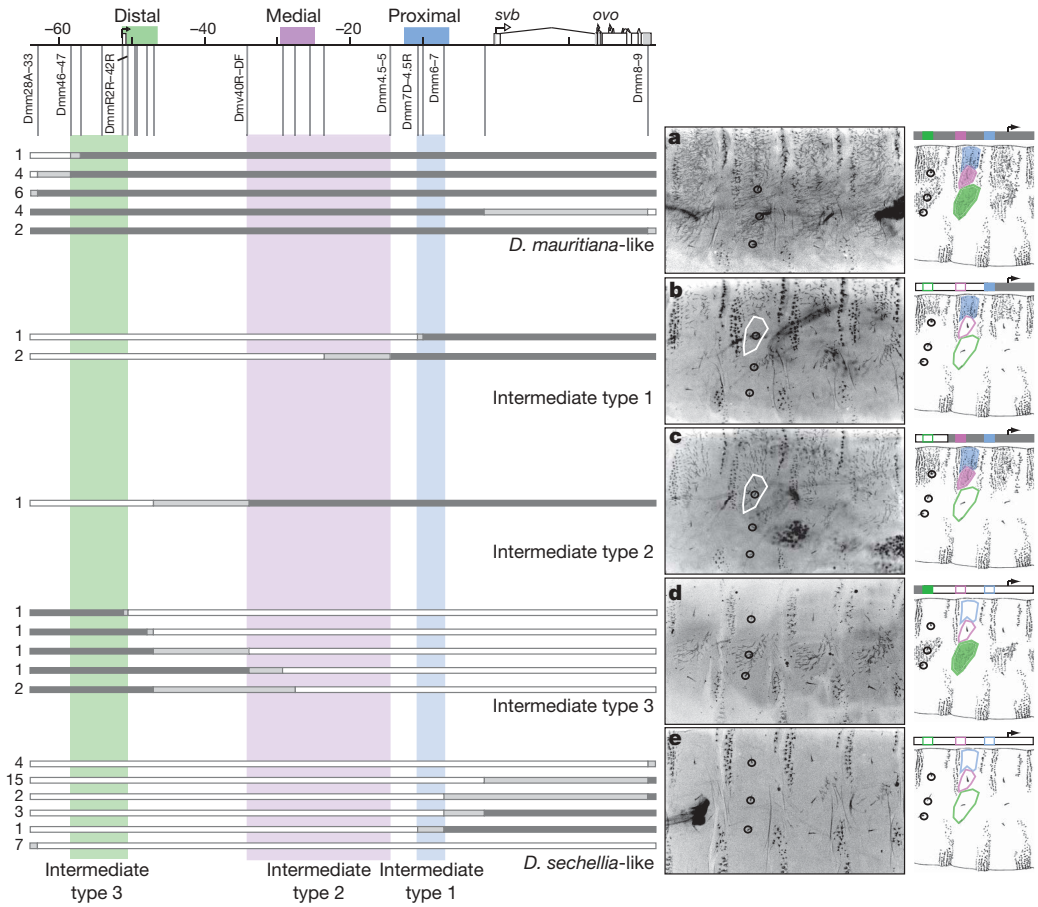

Figure 3 | High-resolution interspecific recombination mapping identifies three enhancer regions of *svb* that caused evolution of the *D. sechellia* trichome pattern. Vertical lines indicate the positions of molecular markers (Supplementary Tables 2 and 3) used to map recombinant chromosomes, grouped according to their trichome pattern. The number of flies carrying a particular recombination breakpoint are indicated on the left. The identity of source DNA in each recombinant chromosome is indicated by the colour of the bar: white, *D. sechellia* DNA; light grey, breakpoint region; darker grey, *D. mauritiana* DNA. **a**, Recombinants that included *D. mauritiana* DNA encompassing the three enhancers produced a *D. mauritiana*-like pattern. **b**, Recombinants containing only the *Dm*_proximal enhancer produced an intermediate type 1 phenotype, characterized by sparse dorsal 4° trichomes. **c**, A recombinant containing the *Dm*_proximal and *Dm*_medial enhancers produced an intermediate type 2 phenotype with dense dorsal and dorsolateral (within white outline) 4° trichomes. **d**, Recombinants containing only the *Dm*_distal enhancer produced intermediate type 3 patterns with lateral patches of 4° trichomes. **e**, Recombinants with *D. sechellia* DNA throughout the *svb cis*-regulatory region produced a *D. sechellia*-like trichome pattern. Diagrams of the trichome patterns are shown at the right, with *D. mauritiana* and *D. sechellia* enhancers indicated as filled and open rectangles, respectively.

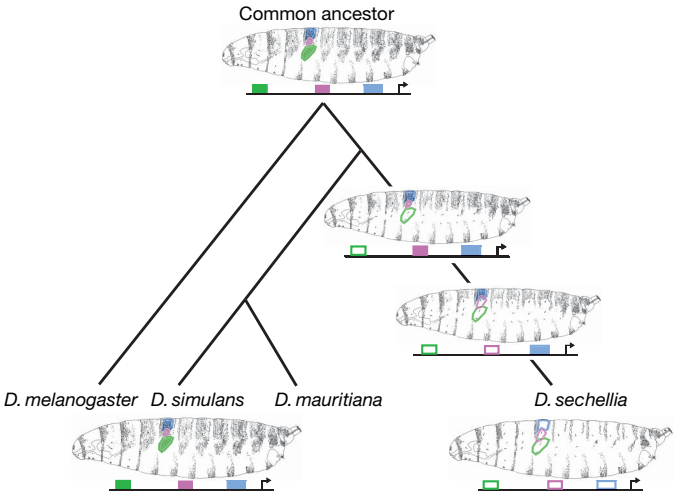

Figure 4 | A possible model of the evolutionary path of the *D. sechellia* trichome pattern. The three *svb* enhancers that drive *D. melanogaster*-like expression in the presumptive common ancestor (top) are indicated as filled rectangles. Each enhancer also drives expression in conserved dorsal and ventral domains, but only the evolving expression patterns are illustrated. At least one change per enhancer region has occurred independently to eliminate *svb* expression progressively in the dorsal and lateral 4° cells as illustrated, or in any other temporal sequence.

METHODS SUMMARY

Reporter constructs. Fragments of *svb* from *D. melanogaster* and *D. sechellia* (Fig. 2 and Supplementary Table 1) were cloned into standard reporter-gene constructs. Expression driven by reporter transgenes was assayed by *in situ* hybridization and by double stainings with an antibody recognizing the reporter gene product and antibodies against endogenous *Drosophila* proteins.

Recombination mapping. *D. mauritiana* flies carrying visible markers flanking the *svb* gene were crossed to *D. sechellia* flies. About 16,000 backcross progeny were examined for the presence of only one of the two flanking markers. About 600 recombinant female progeny were selected, and their wings were removed for genotyping with molecular markers flanking the *svb* locus (Supplementary Table 2). Females with a recombination event in the *svb* gene were then crossed to *D. sechellia* males and the resulting progeny were scored for their larval cuticle phenotypes. Recombination breakpoints within the *svb* gene were mapped by using additional molecular markers (Supplementary Table 2).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions The enhancer analysis was designed by A.P.M., I.D., F.P. and D.L.S.; DNA constructs and transgenic flies were made by A.P.M., I.D. and D.G.S.; embryos were stained, examined and photographed by A.P.M., I.D., J.Z., D.G.S., F.P. and D.L.S.; the recombination mapping experiment was designed and performed by D.L.S., V.O. and A.P.M. All authors participated in data analysis and writing of the manuscript.

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METHODS

Reporter constructs. Fragments of the *svb* gene from *D. melanogaster* and *D. sechellia* were amplified by using the Expand PCR system (Roche) (Fig. 2 and Supplementary Table 1). Map position 0 (Fig. 2) corresponds to the *svb* start codon and X chromosome position 4,894,368 of the *D. melanogaster* genome release 4.3. The *svb* transcription start site was mapped to −250 bp by using 5′-rapid amplification of cDNA ends (data not shown). PCR products were cloned into the pCRII vector (Invitrogen) and then sequenced. Fragments were then subcloned into pCaSpeR-hs43-lacZ[29] or pPTGAL[30] and co-injected with pTURBO[31] into *D. melanogaster* w^{1118} embryos under standard conditions[32]. At least three independent transgenic lines were established for each construct and balanced with the use of *w;Sp/CyO;TM6/DrΔ2-3*.

The *D. melanogaster* proximal construct was made as a *Gal-4* construct, and β-galactosidase expression was driven from a *UAS-lacZ* construct. All other constructs were made as *lac-Z* constructs. Thus, the β-galactosidase expression observed for the *D. melanogaster* proximal construct (Fig. 2d, and Supplementary Figs 4k and 5k) may reflect a delay caused by the initial production of Gal-4 protein. This is supported by examination of the mRNA produced by this construct that reveals expression from the *D. melanogaster* proximal enhancer driven in dorsal 4° cells by stage 14 (Supplementary Figs 3c, f and 4c, g).

Enhancer analysis. Expression patterns driven by reporter constructs were assayed by *in situ* hybridizations with antisense *lacZ* or *GAL4* RNA probes labelled with fluorescein or digoxigenin (Roche). To determine the precise expression domains of the enhancer constructs we performed fluorescent double stainings with an anti-β-Gal antibody (Cappel) and either an anti-Min antibody[33], an anti-Engrailed antibody[34] or an anti-Cut antibody[35]. Embryos were examined on Perkin Elmer RS3 Spinning Disk and Leica TSP2 confocal microscopes.

Fine-scale recombination mapping. We first made a *D. mauritiana* chromosome that carried the 4R1 and 3S1 $white^{+}$ markers (light and dark orange eyes, respectively) flanking the *svb* gene on a $white^{-}$ chromosome. P-element insertions 4R1 and 3S1 correspond to markers 4B(I) and 6BC(I) from ref. 36 and are located in genomic regions AE003432.5 and AE003438.3 (Y. Tao, personal communication), respectively. We crossed these *D. mauritiana* flies with *D. sechellia* $white^{-}$ flies and crossed the F_1 females with *D. simulans* males. We identified female offspring from this cross that carried only one of the two markers on the basis of their eye colour. We then genotyped these recombinants for molecular markers flanking the *svb* locus by using DNA prepared from their wings[37] (Supplementary Table 2). If a female contained a recombination event in the *svb* gene, we crossed her with *D. sechellia* males and scored the resulting progeny for their larval cuticle phenotypes. Half of the larvae were expected to carry the non-recombinant *D. simulans* chromosome and look like *D. simulans*; the other half were expected to carry the recombinant *mauritiana–sechellia* chromosome. Recombination breakpoints within the *svb* gene were mapped by using a further 19 molecular markers (Supplementary Table 2). Larval cuticles were prepared with a 1:1 mixture of Hoyer's medium and lactic acid, and imaged by using phase-contrast or confocal microscopy[38].

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