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LETTERS

Phenotypic robustness conferred by apparently redundant transcriptional enhancers

Nicolás Frankel[1], Gregory K. Davis[2], Diego Vargas[1], Shu Wang[1], François Payre[3] & David L. Stern[1]

Genes include *cis*-regulatory regions that contain transcriptional enhancers. Recent reports have shown that developmental genes often possess multiple discrete enhancer modules that drive transcription in similar spatio-temporal patterns[1–4]: primary enhancers located near the basal promoter and secondary, or 'shadow', enhancers located at more remote positions. It has been proposed that the seemingly redundant activity of primary and secondary enhancers contributes to phenotypic robustness[1,5]. We tested this hypothesis by generating a deficiency that removes two newly discovered enhancers of *shavenbaby* (*svb*, a transcript of the *ovo* locus), a gene encoding a transcription factor that directs development of *Drosophila* larval trichomes[6]. At optimal temperatures for embryonic development, this deficiency causes minor defects in trichome patterning. In embryos that develop at both low and high extreme temperatures, however, absence of these secondary enhancers leads to extensive loss of trichomes. These temperature-dependent defects can be rescued by a transgene carrying a secondary enhancer driving transcription of the *svb* cDNA. Finally, removal of one copy of *wingless*, a gene required for normal trichome patterning[7], causes a similar loss of trichomes only in flies lacking the secondary enhancers. These results support the hypothesis that secondary enhancers contribute to phenotypic robustness in the face of environmental and genetic variability.

The *cis*-regulatory region of the *svb* gene integrates inputs from multiple gene regulatory networks to generate a complex pattern of expression in the embryonic epidermis of insect species[6,8]. SVB protein then activates many downstream genes, ultimately resulting in trichome morphogenesis[9,10]. Three enhancer modules located in a 50 kilobase (kb) region upstream of the *svb* transcription start site (called *7*, *E* and *A*) together recapitulate the complete *svb* epidermal expression pattern[11]. Partial loss of function of all three enhancers led to the evolutionary loss of the long, thin quaternary trichomes (shown in Figs 1a and 2a) on first-instar larvae of *Drosophila sechellia*, a species that is closely related to *Drosophila melanogaster*[11]. Evolution of *svb* expression patterns has probably also contributed to parallel loss of quaternary trichomes in the *Drosophila virilis* group, species of which are distantly related to *D. melanogaster*[12].

We noticed that a 41 kb region upstream of the three known *svb* enhancers displays high conservation among drosophilids, but contains only one small gene named *SIP3* (Fig. 1b and Supplementary Fig. 1). To test whether this region contained additional *svb* enhancers, we assayed reporter constructs encompassing the entire region (Supplementary Fig. 1). Two constructs drove expression in the dorsolateral epidermis in patterns that reproduced part of the native *svb* expression pattern (Fig. 1c, f and Supplementary Fig. 2). To characterize the precise expression domains driven by these newly discovered enhancers, we performed co-immunodetection of the β-galactosidase reporter and of the Dusky-like protein, an early component of developing trichomes[10].

The *Z* enhancer drove expression in many cells that produce quaternary trichomes (Fig. 1c). This expression overlaps the patterns driven by the three enhancers identified previously: *7*, *E* and *A* (Fig. 1b). The *DG2* enhancer drove expression in a more restricted region (Fig. 1f) that overlaps the domain of expression driven by the *E* enhancer. Both *Z* and *DG2* drive expression starting at stage 14 of embryogenesis (Supplementary Fig. 2), which is similar to the time when *svb* mRNA can be detected in epidermal cells.

Given the redundant expression patterns of *Z* and *DG2* with the three enhancers identified previously, we sought further evidence that *Z* and *DG2* encode functional *svb* enhancers. We reasoned that if the *Z* and *DG2* enhancers contribute to trichome patterning, then they should have evolved in a similar way to the previously discovered *7*, *E* and *A* enhancers; they should retain expression in species that also produce quaternary trichomes (such as *Drosophila simulans*), and show reduced expression in *D. sechellia*, which has lost quaternary trichomes. We therefore assayed *Z* and *DG2* enhancer constructs made with orthologous regions from *D. simulans* and *D. sechellia*. These regions were straightforward to identify because the genomes of these species are 3–5% divergent from *D. melanogaster*. The *D. simulans Z* and *DG2* enhancers drove an expression pattern similar to that of the orthologous *D. melanogaster* enhancers (Fig. 1c, d, f, g), which indicates that *Z* and *DG2* contribute to the production of quaternary trichomes both in *D. melanogaster* and in *D. simulans*. In contrast, the *Z* and *DG2* enhancers from *D. sechellia* drove low levels of expression in only a few cells (Fig. 1e, h). The weak expression driven by the *D. sechellia Z* and *DG2* constructs is consistent with the partial loss of expression driven by the *D. sechellia A*, *E* and *7* enhancers and with the loss of quaternary trichomes in this species[11].

To further assess the functional importance of the *Z* and *DG2* enhancers, we generated a 32 kb chromosomal deficiency on the X chromosome that removes both enhancers, called $Df(X)svb^{108}$ (Fig. 1b). As a control, we used strain *C108*, which carries both of the parental transposable elements that were used to generate the deletion. $Df(X)svb^{108}$ flies are viable and display no gross abnormalities. We examined first-instar larvae in detail and found that, when $Df(X)svb^{108}$ embryos developed at the optimal temperature for development (25 °C), larvae exhibited slightly fewer quaternary trichomes (Fig. 2b) and a reduction in the size of the lateral sensory bristles (Supplementary Fig. 3). These results indicate that, under optimal conditions, *Z* and *DG2* are functional enhancers of the *svb* gene that contribute to fine details of trichome patterning and perhaps to bristle morphogenesis. Despite this evidence that the *Z* and *DG2* enhancers contribute to *svb* activity, their loss-of-function phenotype was considerably weaker than one would have expected, given the strong expression

[1]Howard Hughes Medical Institute and Department of Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey 08544, USA. [2]Department of Biology, Bryn Mawr College, 101 N. Merion Ave, Bryn Mawr, Pennsylvania 19010, USA. [3]Université de Toulouse and Centre National de la Recherche Scientifique, Centre de Biologie du Développement, UMR5547, Toulouse, F-31062, France.

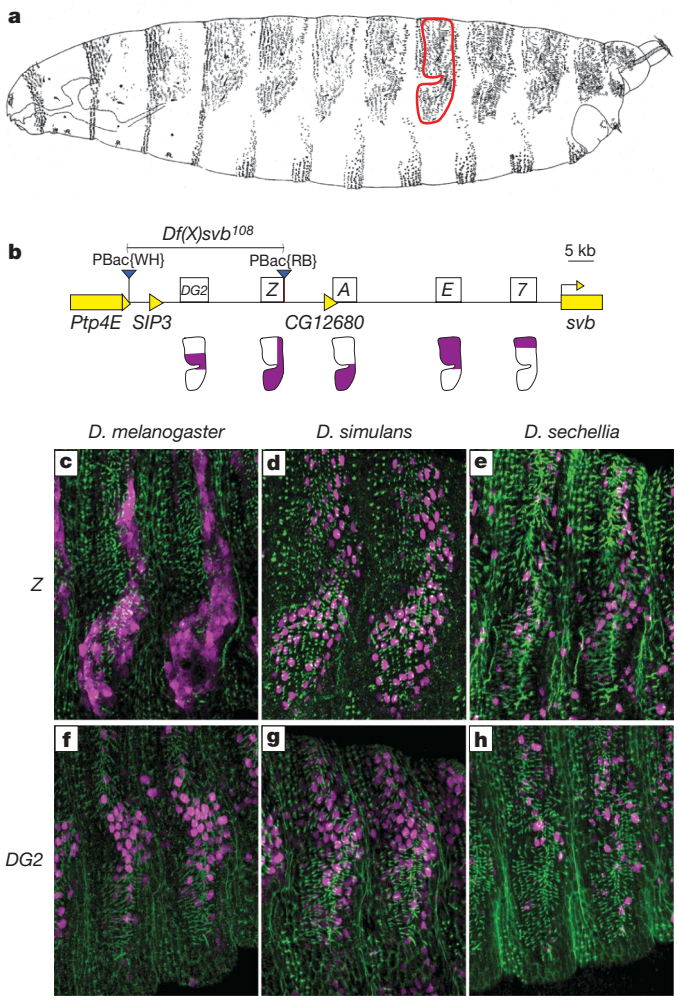

Figure 1 | The *svb* *cis*-regulatory region in *D. melanogaster*. **a**, Drawing from the lateral perspective of a *D. melanogaster* first instar larva. The domain producing quaternary trichomes on the fifth abdominal segment is enclosed in a red outline. **b**, Diagram of the region upstream of the *svb* first exon, showing the positions of the five enhancers for this locus: *DG2*, *Z*, *A*, *E* and *7*. The expression driven by these enhancers in quaternary cells is shown in purple in the diagrams below each enhancer. The piggyBac elements used to generate $Df(X)svb^{108}$ are shown as blue triangles. **c**, **f**, Expression pattern driven by *D. melanogaster Z*::*lacZ* (**c**) and *DG2*::*lacZ* (**f**) in the 5th and 6th abdominal segments of a stage-15 embryo (purple). An anti-Dusky-like antibody was used to stain developing trichomes (green). **d**, **g**, Expression pattern driven by *D. simulans Z*::*lacZ* (**d**) and *DG2*::*lacZ* (**g**). **e**, **h**, Expression pattern driven by *D. sechellia Z*::*lacZ* (**e**) and *DG2*::*lacZ* (**h**). β-galactosidase protein produced by *D. melanogaster Z*::*lacZ* is expressed in the cytoplasm; β-galactosidase from all other constructs is localized to the nucleus.

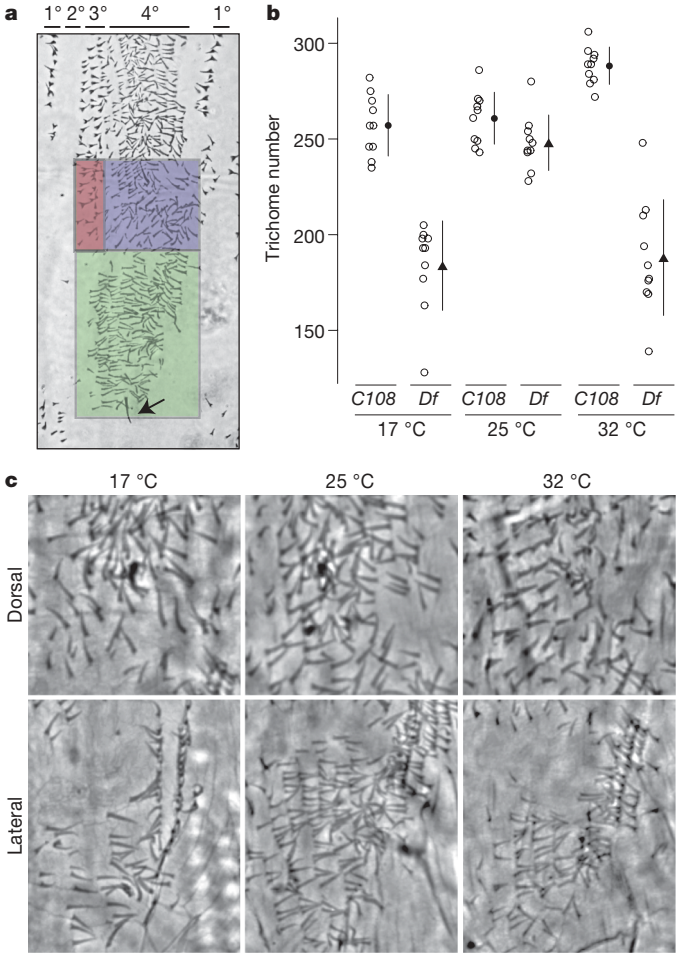

Figure 2 | Effect of $Df(X)svb^{108}$ on the number of quaternary trichomes. **a**, The lateral patch (green) and dorsal region (blue) in which trichomes were counted. The green and blue boxes correspond to the regions where the *Z* and *DG2* enhancers are expressed strongly. The primary, secondary, tertiary and quaternary cell types are indicated with horizontal lines above the photograph. The arrow marks the spiracle that was used to set the lower boundary for the green box. The blue box was positioned directly above the green box. The red box identifies the stout tertiary trichomes, which were excluded from the counts. **b**, Number of trichomes in the lateral plus dorsal region (blue and green boxes) of the fifth abdominal segment of the larva. Open circles give trichome numbers for each individual ($n = 10$); the black symbols and lines show the mean ± 1 s.d. Embryos from each of the two genotypes (*C108* and $Df(X)svb^{108}$) were reared at three different temperatures: 17, 25 and 32 °C. **c**, Cuticle images showing the quaternary trichomes in the lateral patch (below) and dorsal region (above) of $Df(X)svb^{108}$ first-instar larvae that developed at the three different temperatures. The genotype by temperature interaction term of a two-way ANOVA was highly significant ($F = 27.57$, $P < 0.0001$).

driven by these enhancers. We reasoned that this resulted from the fact that the *Z* and *DG2* enhancers drive overlapping expression with the enhancers *7*, *E* and *A*, and that the latter three enhancers drive expression levels that are sufficient to generate most larval trichomes when embryos develop under optimal conditions[11].

We therefore considered the hypothesis that *Z* and *DG2* contribute to phenotypic robustness. Natural populations experience repeated stresses over evolutionary time, including variable temperatures. Temperature influences membrane fluidity, enzymatic activity, protein folding, protein–protein interactions, and protein–DNA interactions[13,14]. Organisms have evolved developmental mechanisms to buffer the phenotype in the face of temperature-induced cellular changes. We reasoned that sub-optimal temperatures might destabilize the transcriptional output of genes during embryogenesis and that secondary enhancers may confer a selective advantage by maintaining transcription above a required minimum threshold. We therefore tested the effect of $Df(X)svb^{108}$ in embryos that had developed at 17 and 32 °C, temperatures close to the extremes at which *Drosophila* embryos survive[15]. We counted the number of quaternary trichomes in the regions where *Z* and *DG2* are expressed strongly (Fig. 2a). The *svb* gene is an ideal target for this analysis, because quantitative changes in SVB level influence trichome density, size and shape[16].

Control embryos reared at all temperatures produced similar numbers of trichomes, implying that the number of trichomes is canalized against temperature variation[17]. The number of trichomes on $Df(X)svb^{108}$ larvae reared at 25 °C was similar to the number on control *C108* larvae at all temperatures (Fig. 2b). In contrast, $Df(X)svb^{108}$ larvae displayed a highly significant decrease in trichome numbers when reared at extreme temperatures (Fig. 2b). The primary and tertiary trichomes look normal on $Df(X)svb^{108}$ larvae at all temperatures (data not shown), which is expected, because the *Z* and *DG2*

enhancers do not drive expression in cells producing primary and tertiary trichomes.

In principle, the loss of trichomes observed on *Df(X)svb*^{108} larvae reared at extreme temperatures may have resulted from mechanisms acting independently of the *Z* and *DG2* enhancers. If the effects observed with *Df(X)svb*^{108} resulted from loss of the *Z* and *DG2* enhancers, then reintroducing a functional *Z* or *DG2* enhancer into a *Df(X)svb*^{108} background should rescue some trichomes. We tested this hypothesis for the *Z* enhancer. We generated a transgene carrying the *svb* cDNA under the transcriptional control of the *Z* enhancer and introduced it onto the third chromosome of *Df(X)svb*^{108} flies. At extreme temperatures, the *Z::svb* cDNA transgene completely rescued wild-type trichome numbers in the lateral patch (Fig. 3a and Supplementary Fig. 3). However, in the region dorsal to the lateral patch, the rescue is very weak or absent (Fig. 3b and Supplementary Fig. 3). This is consistent with the fact that *Z* drives expression at high levels in the lateral region, where rescue is observed, and only weakly in a small number of cells of the dorsal region (Fig. 1). The loss of canalization in the dorsal region of *Df(X)svb*^{108} larvae may be caused by loss of *DG2*, which drives expression mainly in this dorsal region. These results demonstrate that *Z* contributes to phenotypic robustness. Moreover, the rescue of trichome numbers by a transgene introduced onto a different chromosome from the *svb* locus indicates that *Z* does not need to be in intimate contact with other *svb* enhancers or with the *svb* basal promoter to buffer *svb* function. Instead, we propose that *Z* contributes to phenotypic robustness simply by boosting levels of *svb* transcription in the cells in which *Z* drives expression.

Given this evidence that the *Z* enhancer, and possibly also *DG2*, contributes to robustness against environmental perturbations, we asked whether these enhancers also buffer against genetic perturbations. For example, it has been reported previously[18] that two Dorsal target genes that possess 'shadow' enhancers maintain synchronous transcriptional activation across *Dorsal*$^{+/-}$ embryos, whereas two Dorsal target genes that seem to lack such 'shadow' enhancers display less synchrony in *Dorsal*$^{+/-}$ embryos. Therefore, we tested the effect of reducing Wingless signalling, which is required for normal development of quaternary trichomes[7], by crossing the *Df(X)svb*^{108} allele and the *C108* control allele into a background heterozygous for a *wingless* null allele. At 25 °C, the *Df(X)svb*^{108};*wg*$^{-/+}$ embryos produced significantly fewer trichomes than *C108*;*wg*$^{-/+}$ embryos, *Df(X)svb*^{108} embryos, and *C108* embryos (Fig. 4). The combined results indicate that the *Z* and *DG2* enhancers buffer against both environmental and genetic perturbations.

These results indicate that the production of larval trichomes is normally canalized and that this is accomplished, at least in part, through transcriptional activation mediated by the *svb* secondary enhancers that are removed in *Df(X)svb*^{108}.

The *svb* locus contains multiple enhancers with overlapping expression patterns. Similar patterns of overlapping enhancer activity have been found for the *cis*-regulatory regions of the *Drosophila* genes *sog* (ref. 1), *vnd* (ref. 3) and *brinker* (ref. 1) and for the *cis*-regulatory regions of the mouse genes *sonic hedgehog* (ref. 4) and *sox10* (ref. 2). Moreover, it has been estimated that 50% of the target genes of the transcription factor Dorsal contain shadow enhancers[5]. Therefore, the presence of additional enhancers in *cis*-regulatory regions may be a common signature of developmental regulators. This may explain why, in previous reports, animals carrying deletions of highly conserved enhancers have not displayed observable phenotypic defects when reared in standard laboratory conditions[19,20].

Developmental buffering is likely to result from many molecular mechanisms. For example, deletion of the conserved miRNA *miR7* in *D. melanogaster* has no obvious phenotypic effect in normal laboratory conditions, but it is required to canalize the expression of the gene

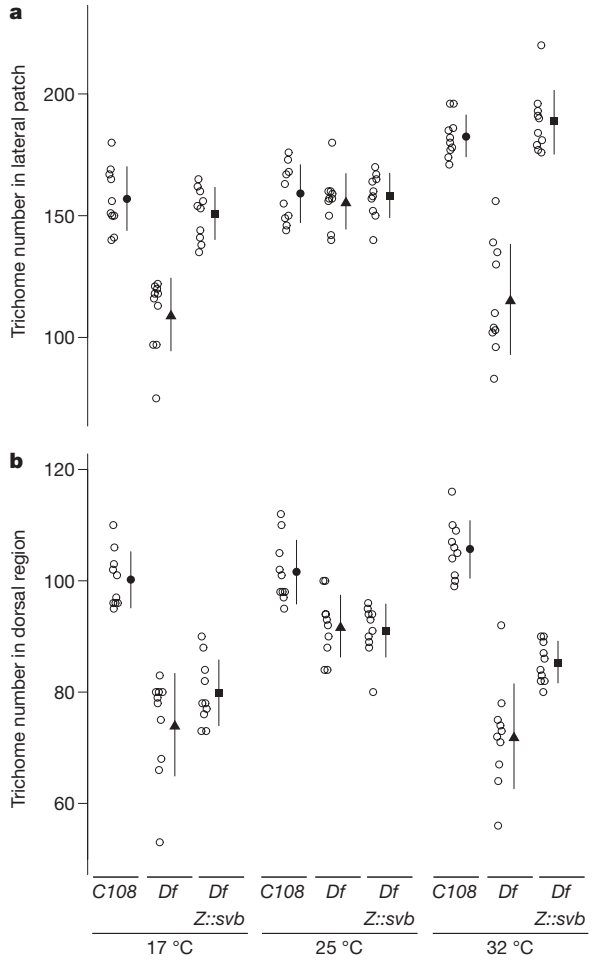

Figure 3 | Rescue of the temperature-dependent trichome loss in the lateral patch by a *Z::svb* transgene. **a**, **b**, Trichome number in the lateral patch (**a**) and dorsal region (**b**) of the fifth abdominal segment of larvae with the genotypes *C108*, *Df(X)svb*^{108} and *Df(X)svb*^{108}; *Z::svb*. Open circles represent trichome numbers for each individual ($n = 10$); the black symbols and lines show the mean ± 1 s.d.

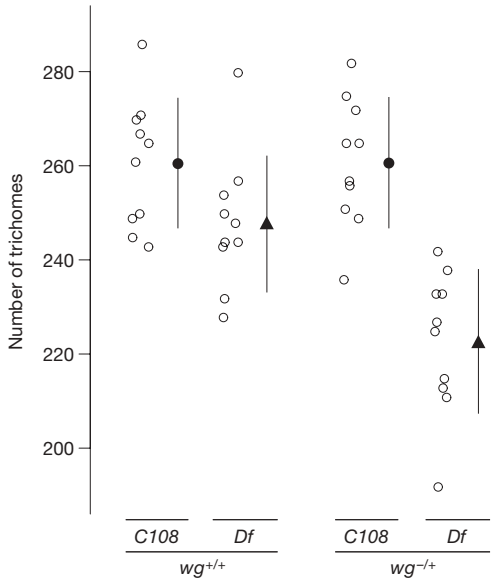

Figure 4 | Effect of *Df(X)svb*^{108}; *wg*$^{-/+}$ on the number of quaternary trichomes. *C108* and *Df(X)svb*^{108} embryos that were heterozygous for a null allele of *wingless* were reared at 25 °C. Quaternary trichomes were counted as described in the legend to Fig. 2. A two-way ANOVA shows a highly significant genotype by temperature interaction ($F = 7.79$, $P = 0.0084$), which is caused by a large reduction in the number of trichomes on *Df(X)svb*^{108}; *wg*$^{-/+}$ larvae relative to all other genotypes.

atonal under fluctuating temperatures[21]. Similarly, our results indicate that *svb* secondary enhancers have a minimal role at optimal conditions for development, but that they are essential to buffer the trichome phenotype under genetic or environmental variability. Secondary enhancers are likely to be evolutionarily maintained by selection for robustness against temperature fluctuation, genetic background effects[22] and expression noise[23].

METHODS SUMMARY

The target regions were PCR-amplified from genomic DNA from *D. melanogaster*, *D. simulans* and *D. sechellia*. These PCR fragments were cloned into pCaSpeR-hs43-lacZ or placZattB and integrated into the *D. melanogaster* genome to test their enhancer activity. The precise expression domains of the enhancer constructs were determined by double staining with a mouse anti-β-galactosidase antibody (Promega) and a rabbit anti-Dusky-like antibody[10] and then by examining stained embryos with a confocal microscope. $Df(X)svb^{108}$ was generated via flippase-induced deletion of the DNA between two FRTs (flippase recognition targets) present in *C108*. We made 0–3 h embryo collections and reared embryos to hatching at different temperatures. First-instar larvae were mounted in 1:1 Hoyer's:lactic acid mixture and cuticles were imaged with phase-contrast microscopy. Trichomes were counted using ImageJ. A null allele of *wingless* (wg^{IG22}, ref. 24) was used to obtain males with the genotypes $Df(X)svb^{108}$/Y, $+/wg^{IG22}$ and *C108*/Y, $+/wg^{IG22}$.

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

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METHODS

Reporter constructs. Genomic DNA from *D. melanogaster*, *D. simulans* and *D. sechellia* (see primer list below) was amplified using the Expand HiFi PCR system (Roche) and cloned into pGEMT Easy (Promega). Fragments *Z* from *D. melanogaster* and *Zprox* from *D. simulans* were subcloned into pCaSpeR-hs43-lacZ using NotI. This plasmid was co-injected with pTURBO33 into *D. melanogaster* w^{1118} embryos using standard conditions. At least three independent transgenic lines were established for each construct. The remaining fragments were subcloned into placZattB using NotI and injected into line *M{3xP3-RFP.attP} ZH-51D* (with *M{vas-int.Dm}ZH-2A*)[25].

Primers used were for *D. melanogaster DGO*: forward 5′-TGGCCTGTGCCATGTGTGCGAGTACG-3′, reverse 5′-TGGGTGCGCAATTATGCCGCCAGAGC-3′; *D. melanogaster DG1*: forward 5′-CTGGGTGTGTGTGCAATATGTGAGC-3′, reverse 5′-GTGAGGGTACAAGGCGAAATCGAAA-3′; *D. melanogaster DG2*: forward 5′-AATTGTTCGCACGCTTCGCTCTAA-3′, reverse 5′-GATTGGTGCCGAGAGGTGAAAGTG-3′; *D. melanogaster DG3*: forward 5′-GGCCACAACTCAATGGCAAAAATG-3′, reverse 5′-CAGCAGCGAATCAAGACGAAAGGT-3′; *D. melanogaster DG4*: forward 5′-CCCCCGTCTTTGTCTGTTTGTCTG-3′, reverse 5′-GGAACACAATCTGCCTGCCTGACT-3′; *D. melanogaster DG5*: forward 5′-TATCCTTTTACGACGCCCCTGTGTC-3′, reverse 5′-GATTCGGTTCCTTGGGATTGGATTT-3′; *D. melanogaster Z*: forward 5′-ATTGCTTCGGCTCTCCCGTTA-3′, reverse 5′-TTGTGTGGCTCACTTGGCAC-3′; *D. simulans Zprox*: forward 5′-GTGAAAGATCGGATCCGTCT-3′, reverse 5′-GTTCGTATCGCCCACTTGAAT-3′; *D. simulans Z*: forward 5′-ATTGCTTCGGCTCTCCCGTTA-3′, reverse 5′-TTATGTGGCTCACTTGGCAC-3′; *D. sechellia Z*: forward 5′-ATTGCTTCGGCTCTCCCGTTA-3′, reverse 5′-TTGTGTGGCTCACTTGGCAC-3′; *D. simulans DG2*: forward 5′-TGCTTTTCCAACCCCTCAGTT-3′, reverse 5′-GGGGGTGCAGGCTATTTTGTTC-3′; *D. sechellia DG2*: forward 5′-TGCTTTTCCAACCCCTCAGTT-3′, reverse 5′-GAGGGTGCAGGCTATTTTGTTC-3′.

Only transgenes containing the *Z* and *DG2* regions drove expression in the dorso-lateral epidermis. *DG3*, which is contained within the region deleted by *Df(X)svb*^{108}, drove weak expression in the ventral epidermis, but no phenotypic changes in the ventral denticles were observed at any temperature. *Zprox* was analysed from *D. simulans* DNA, as this region lacked a large *roo* element that is present in the *D. melanogaster* genome.

Immunohistochemistry and immunofluorescence. Embryos were fixed using standard conditions. To determine the precise expression domains of the enhancer constructs we performed fluorescent double staining with a mouse anti-β-galactosidase antibody (Promega) and a rabbit anti-Dusky-like antibody[10]. Alexa-488 anti-rabbit and Alexa-647 anti-mouse (Molecular Probes) were used as secondary antibodies. The embryos were examined on a Leica TCS SPE confocal microscope. For immuno-histochemistry, we used a rabbit anti-β-galactosidase antibody (Cappel) and anti-rabbit antibody coupled to horseradish peroxidase (Santa Cruz Biotech) and staining was developed with DAB/Nickel.

Generation of *Df(X)svb*^{108}. pBac{WH}Ptp4E[f02952] and pBac{RB}e03292 were recombined onto the same X chromosome and a homozygous stock was generated (named *C108*). This stock was crossed to a line containing a *hs*::*flipase* and larvae were heat shocked at 37 °C for 1 h each day during larval development. After crossing these adults to *white*$^{-}$ flies, we selected adults that had lost one copy of the *white*$^{+}$ transgene (originating on one of the pBac transgenes), which is expected if the two FRT sites recombined to generate a deletion. The deletion was confirmed by a PCR experiment, which amplified a fragment containing a chimaeric piggyBac element. The primer used (5′-TGCATTTGCCTTTCGCCTTAT-3′) amplified the expected 7.3 kb fragment[26]. We then generated a stock homozygous for the deletion. This allele is named *Df(X)svb*^{108}.

Embryo collection and cuticle microscopy. We made 0–3 h embryo collections (many hours before the onset of *svb* expression in epidermal tissues) and transferred embryos to dishes with water at the different temperatures. Two days later, we collected first instar larvae and incubated them at 60 °C for 4 h. Subsequently, larvae were mounted on a microscope slide with a drop of 1:1 Hoyer's:lactic acid mixture. After overnight drying, the cuticles were imaged with phase-contrast microscopy.

Trichome counting. A spiracle below the lateral patch was used as a landmark to position the green box (shown in Fig. 2a). The blue box was positioned directly above the green box (shown in Fig. 2a). Both boxes were programmed as macros in Image J software (http://rsb.info.nih.gov/ij/). The trichomes were counted using the cell-counter option of Image J.

Rescue experiments. The cDNA of *svb* was amplified from the plasmid pUAS-svb (ref. 6) with primers NsiI-svbcDNAfw (5′-ATGCATTTAACTCACCTGGGCGAATCC-3′) and NdeI-svbcDNArv (5′-CATATGTTGCAGCTTGTTCGGTTGGTA-3′) and cloned into pCR-Blunt II-TOPO (Invitrogen). The *svb* cDNA was subcloned with NsiI and NdeI into a version of placZattB (ref. 25) that had the *lacZ* removed (by cutting with PstI and NdeI). We named this plasmid pRSQsvb. The *Z* enhancer was amplified with the primers used previously (see reporter constructs) that had the addition of 3′ XbaI sites. This PCR fragment was cloned into pGEMT (Promega) and subcloned into pRSQsvb using XbaI. This plasmid was injected into the recipient line *M{3xP3-RFP.attP}ZH- 86Fb* (with *M{vas-int.Dm}ZH-2A*)[25]. A third chromosome carrying the Z::*svb* transgene was introduced into the *Df(X)svb*^{108} line to obtain a stock homozygous for both the deficiency, on the X chromosome, and Z::*svb*, on the third chromosome, and is referred to as *Df(X)svb*^{108}; *Z*::*svb*.

***Wingless* experiment.** A null allele of *wingless* (*wg*^{IG22}, ref. 24) was used to obtain males of the genotype FM7c, actin::GFP/Y; CyO, actin::GFP/*wg*^{IG22}. These males were crossed to females with either *Df(X)svb*^{108}/*Df(X)svb*^{108} or *C108*/*C108* genotypes. We selected for non-fluorescent progeny, which were male first-instar larvae heterozygous for the *wingless* null allele: *Df(X)svb*^{108}/Y; *wg*$^{IG22/+}$ or *C108*/Y; *wg*$^{IG22/+}$.

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