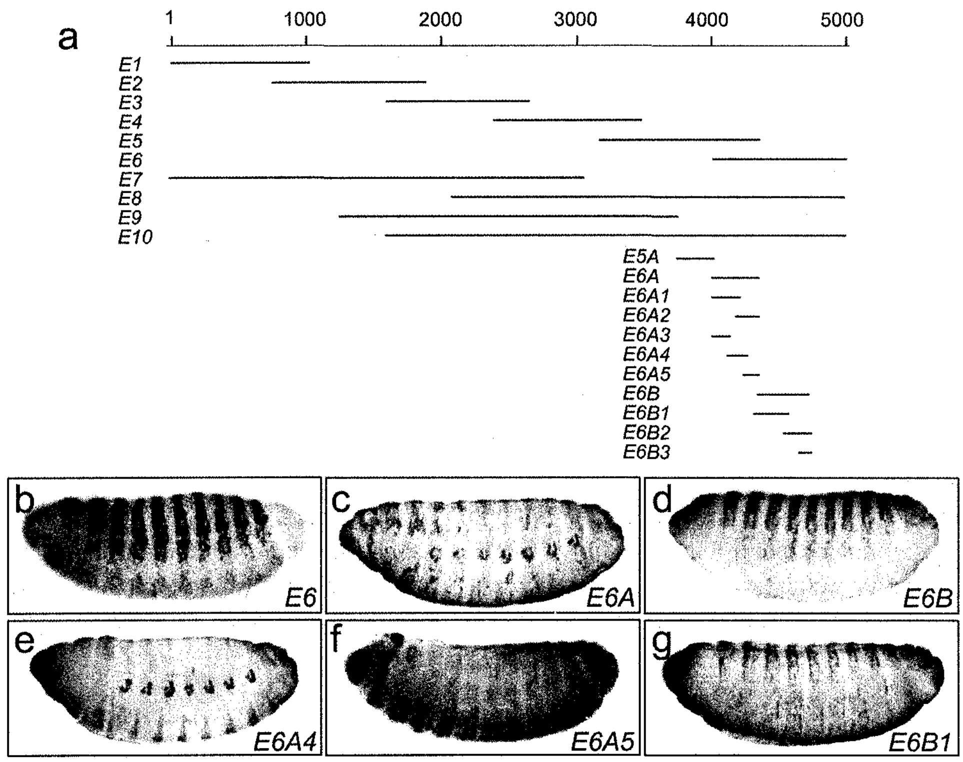
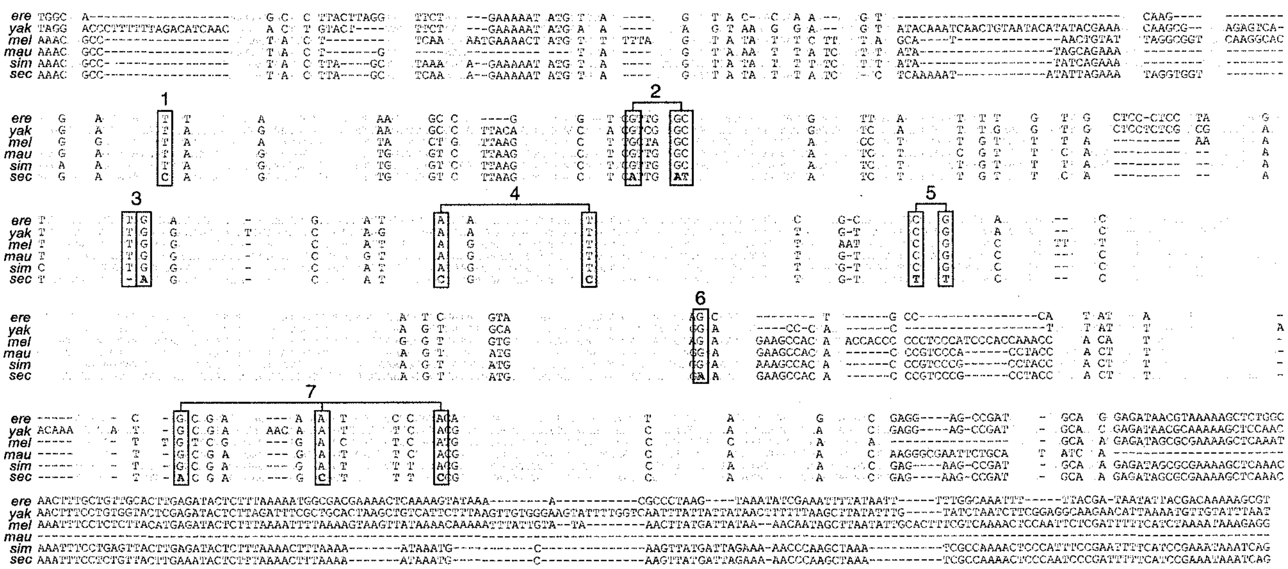
doi:10.1038/nature10200



Supplementary Figure 1. Dissection of the *Drosophila svb E* region. (**a**,**b**) We first divided the *E* region into 10 overlapping fragments (*E1* - *E10*). Regions *E3* and *E6* drove strong expression that recapitulated the complete expression pattern driven by *E* (**b** and Figure 1h,i). Region *E2* drove an expression pattern that matched part of the ventral pattern driven by *E3* (not shown). We then divided the *E6* region into ten overlapping fragments and tested a region of *E5* that abuts *E6*. We did not detect expression from the *E5A*, *E6A1*, *E6A2*, *E6A3*, and *E6B2* constructs. (**c**,**d**) The *E6A* (**c**) and *E6B* (**d**) fragments drove expression in apparently complementary domains. (**e-g**) Smaller fragments from these regions revealed three smaller pieces that gave weak expression; *E6A4* (142bp) (**e**), *E6A5* (105bp) (**f**), and *E6B1* (239bp) (**g**). The expression of *E6A5* appears to be ectopic, since we could not detect expression until stage 15, and expression was observed mainly in the ventral half of the embryo. The *D. simulans E6A4*, *E6A5*, and *E6B1* regions generated expression patterns that could not be distinguished from the orthologous *D. melanogaster* regions (not shown). However, we could not detect any expression from the orthologous *D. sechellia* fragments (not shown).



Supplementary Figure 2. Sequence alignment of the *E6* region from *D. erecta* (*ere*), *D. yakuba* (*yak*), *D. melanogaster* (*mel*), *D. mauritiana* (*mau*), *D. simulans* (*sim*), and *D. sechellia* (*sec*). Conserved bases are shown in grey. Positions of the alignment with *D. sechellia*-specific changes are enclosed in rectangles and the *D. sechellia*-specific nucleotides are red. Numbers indicate the seven clusters of mutations that were engineered into the *E10* constructs.

Methods and Materials

Reporter Constructs

Fragments of the E enhancer were amplified from genomic DNA of *D. melanogaster* w^{118} using the primers listed below and subcloned into either pCaSpeR-hs43-lacZ or placZattB. Recombinant pCaSpeR plasmids were co-injected with pTURBO33 into *D. melanogaster* w^{1118} embryos using standard conditions. At least three independent transgenic lines were established for each construct. Recombinant pLacZattB constructs were injected into line M(3xP3-RFP.attP)ZH-51D; M(vas-int.Dm)ZH-2A. Regions E6A4, E6A5, and EB1 were cloned also from *D. sechellia* 14021-0248.28 and from *D. simulans* w^{501} (see Supp. Fig. 1).

Region	Forward Primer	Reverse Primer
E1	DmEf CACTGTACATCTCGGATTTGC	E1R CGTCGACAGGCGACAAGTG
E2	E2F GCAAACACACGATTCTAGCCGA	E2R AACTAAACGATCGCACCGGAT
E3	E3F AGATCGAAGACAAATTATGCTGAT	E3R CTTCAATGGCCCATTGAAATGAC
E4	E4F CCATCCTCCGCAGTTGGATAA	E4R TCATTCGCTCTGTCGACCTGT
E5	E5F AAAATGTAATTAACCTCAAGTTTCCTT	E5R TGTCCACTGCACTAATCTAGCATT
E5A	E5AF TCTTCCAGTTGGACGAACTCA	E5AR TAAGCCATTAGTTTTGGCTTGTTT
E6	E6F AAACAAGCCAAAACTAATGCCTTA	DmER GCTTAACAAGGTATGACAATCCAT
E6A	E6F AAACAAGCCAAAACTAATGCCTTA	E5R TGTCCACTGCACTAATCTAGCATT
E6A1	E6F AAACAAGCCAAAACTAATGCCTTA	E6A3R GGTGGAATCCTTAAGTTGG
E6A2	E6A3F CCAACTTAAGGATTCCACC	E6A5R TGTCCACTGCACTAATCTAG
E6A3	E6F AAACAAGCCAAAACTAATGCCTTA	E6A2R AGTGCCTTGTGCCTTGTGA
E6A4	E6A2F TCACAAGGCACAAGGCACT	E6A4R TAACATACTTAAATAATGGGCTT
E6A5	E6A4F	E6A5R

	AAGCCCATTATTTAAGTATGTTA	TGTCCACTGCACTAATCTAG
E6B	E6BF AATGCTAGATTAGTGCAGTGGACA	E6BR ATCCATATCTGGTTGCCTCATA
E6B1	E6BF AATGCTAGATTAGTGCAGTGGACA	E6B2R TGGCTTCGAATTTTCTTCAG
E6B2	E6B2F CTGAAGAAAATTCGAAGCCA	E6BFR CATATCTGGTTGCCTCATATG
E7	DmEf CACTGTACATCTCGGATTTGC	E7R TAAATGGACGCACTTGGAGCT
E8	E8F TCGCGGCTTACCCAGTGCA	DmER GCTTAACAAGGTATGACAATCCAT
E9	E9F GCCGACCGCAGCAAATGACT	E9R TGAGTTCGTCCAACTGGAAGA
E10	E3F AGATCGAAGACAAATTATGCTGAT	DmER GCTTAACAAGGTATGACAATCCAT
D.simulans E6	E6F AAACAAGCCAAAACTAATGCCTTA	sim/sechE6BFr CAGATCTGGTTGCCTCATATG
D. sechellia E6	E6F AAACAAGCCAAAACTAATGCCTTA	sim/sechE6BFr CAGATCTGGTTGCCTCATATG
D. simulans E6A4	simE6A2f TTCAAAGACACTAGCAAATAAT	simE6A4r TTTAACATACTTAAATAATGGACTT
D. sechellia E6A4	sechE6A2f TTCAAAGGCACTAGCAAATAA	sechE6A4r" GTTTAACATACTTAAATAATGGACTT
D. simulans E6A5	sechE6A4f" GTTTAACATACTTAAATAATGGACTT	simE6A5r CCACTGCACTAATCTAGC
D. sechellia E6A5	sechE6A4f" GTTTAACATACTTAAATAATGGACTT	sechE6A5r TGGCCACTGCACTAATCTAG
D. simulans E6B	simE6Bf AATGCTAGATTAGTGCAGTGGACA	sim/sechE6BFr CAGATCTGGTTGCCTCATATG
D. sechellia E6B	simE6Bf AATGCTAGATTAGTGCAGTGGACA	sim/sechE6BFr CAGATCTGGTTGCCTCATATG
D. simulans E6B1	sechE6Bf AATGCTAGATTAGTGCAGTGGCCA	simE6B2r TGGCTTTGAATTTTCCTCAG
D. sechellia E6B1	sechE6Bf AATGCTAGATTAGTGCAGTGGCCA	sechE6B2r TGGCTTCGAATTTTTCTCAG

Immunohistochemistry and in situ *hybridization*

Embryos from transgenic lines were fixed using standard conditions and ß-Gal expression was detected with immuno-histochemistry using a rabbit anti-βGal antibody (Cappel) used at 1:2000 and and anti-rabbit antibody coupled to HRP (Santa Cruz Biotech), also used at 1:2000. Staining was developed with DAB/Nickel.

To detect the expression of transgenic *svb* transcripts, we made a RNA probe complementary to the *lacZ* and *SV40* sequence in the 3' UTR of the *svb* cDNA using the

Dig RNA labeling kit (Roche). The probe was subjected to base hydrolysis for 15 minutes, and passed over a G50 spin column to remove unincorporated nucleotides (Pharmacia, Kalamazoo, MI). For hybridization and staining, we adapted a standard *in situ* protocol[1] for use in an Intavis InsituPro Vsi (Chicago, IL) instrument. We replaced the gentle rocking steps with frequent aspiration/dispensation cycles during washes; the Intavis pipettor was programmed to aspirate and dispense solution from all samples, one at a time, in a continuous loop. During hybridization with the RNA probe and during incubation with the anti-Dig antibody, we agitated samples by aspiration/dispensation once each hour. Prior to proteinase K digestion, we permeabilized embryos by stepping from 100% EtOH to 25:75 Xylene:EtOH to 50:50 Xylene:EtOH for one hour each. The anti-Dig antibody (Roche, Indianapolis, IN) was preabsorbed with fixed *D. melanogaster* embryos at a 1:10 concentration prior to use, then used at a final concentration of 1:2000 overnight in an antibody solution containing Roche Blocking Solution (Indianapolis, IN).

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. The *mut_All* embryos were compared with the *E10* construct at stage 13, the *mel_mut* constructs were compared with *mel_E10* at stage 12, and the *sec_mut* embryos were compared with *sec_E10* at stage 14. We then tested for a difference in the proportion of stained embryos with the Barnard test for 2x2 contingency tables[2,3] using a Matlab routine[4]. We then applied a sequential Bonferroni correction for multiple tests[5,6] to identify individual tests that were significant at the 0.05 and 0.01 level. The test statistics and uncorrected P values are shown in Supplementary Table 1.

Trichome rescue experiments

We cloned *D. melanogaster* and *D. sechellia E10* into pRSQsvb[7]. *D. melanogaster E10* was amplified using primers XbaI-E10fw (<u>TCTAGA</u>AGATCGAAGACAAATTATGCTGATC) and XbaI-DmelE10rv (<u>TCTAGA</u>AGCTTAACAAGGTATGACAATCCAT). This PCR fragment was cloned into pGEMT (Promega) and subcloned into pRSQsvb using XbaI. *D. sechellia* E10 was amplified with primers XbaI-E10fw (TCTAGAAGATCGAAGACAAATTATGCTGATC) and XbaI-DsechE10rv (TCTAGAATCTGAACGAGGTATGACAATCCAT) and cloned into pRSQsvb using the above strategy. Mutant plasmids were generated using site-directed mutagenesis (Genescript USA Inc.). These plasmids were injected into the recipient line M(3xP3-RFP.attP)ZH- 86Fb; M(vas-int.Dm)ZH-2A. Males homozygous for the transgene were crossed to svb^{R9} /FM7c;*twi::GFP* females[8]. Thus, we could analyze trichome rescue by the transgenes in a *svb* null background. We made overnight embryo collections and transferred embryos to plastic petri dishes containing distilled water and maintained them in a 25°C incubator. Two days later, we collected non-fluorescent first instar larvae and incubated them at 60°C for 4 hours. Subsequently, larvae were mounted on a microscope slide in a drop of 1:1 Hoyer's:lactic acid mixture. After overnight drying, the cuticles were imaged with phase-contrast microscopy.

Trichome counting

The dorsal midline was used as a landmark to position the top of a rectangle in the dorsal region and a second rectangle was positioned directly below the first, thus defining the dorsal and lateral regions (Fig. 5c). Both rectangles were programmed as macros in Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009). The trichomes were counted using the cell-counter option of Image J. We performed pairwise comparisons of trichome numbers between the wild type construct and each mutated construct. Statistical significance was assessed with Dunnet's test and the ANOVA results are reported in Supplementary Table 2.

Amplification, sequencing and analysis of svb *cis-regions from* D. simulans *and* D. sechellia

Genomic DNA was extracted from *D. simulans* w^{501} and *D. sechellia* 14021-0248.28 (Drosophila Species Stock Center). Two PCR fragments flanking the E6 region were amplified using primers E6flank1fw (TGCCGATTGCCATTTTGGTGGC) and E6flank1rv (TCGCGCTATCTCGTGTTGCGG), and E6flank2fw (TGGACGAACTCAACGGAAGCAACG) and E6flank2rv (CCACCAGGCCACTGCAGCAA) The purified PCR products were sequenced with internal primers every 800 bp (the sequence is available upon request). Substitution rates were computed with MEGA 4 software[9]. *D. sechellia E6* was sequenced from lines 14021-0248.03, 14021-0248.07, 14021-0248.08, 14021-0248.11, 14021-0248.13, 14021-0248.15, 14021-0248.27, 14021-0248.28 and 14021-0248.30, which were obtained from the Drosophila Species Stock Center.

Supplementary Table 1. Results of Barnard test for 2X2 contingency tables[2,3] for changes in the onset of expression in evolved and engineered constructs, calculated using a Matlab routine[4].

Treatment 1 (N)	Treatment 2 (N)	Embryonic stage of comparison	Wald statistic	Nuisance Parameter	P value[1]
sec_E10 (50)	*mel_E10* (31)	13	9.0000	0.6201	0.0000
mel_mut_All (12)	*mel_E10* (31)	13	4.2442	0.0801	0.0005
sec_mut_All (24)	*mel_E10* (31)	13	8.6023	0.6801	0.0000
mel_mut_1 (16)	*mel_E10* (36)	12	0.2830	0.9601	0.5253
mel_mut_2 (11)	*mel_E10* (36)	12	3.0938	0.2101	0.0017
mel_mut_3 (28)	*mel_E10* (36)	12	3.9365	0.2901	0.0000
mel_mut_4 (85)	*mel_E10* (36)	12	3.8007	0.3301	0.0001
mel_mut_5 (28)	*mel_E10* (36)	12	4.4813	0.4501	0.0000
mel_mut_6 (7)	*mel_E10* (36)	12	3.2801	0.2701	0.0008
mel_mut_7 (13)	*mel_E10* (36)	12	1.6372	0.1201	0.0703
mel_mut_All (12)	*sec_E10* (50)	13	5.2610	0.9501	0.0001
sec_mut_All (24)	*sec_E10* (50)	13	8.6023	0.6801	0.0000
sec_mut_1 (130)	*sec_E10* (52)	14	1.2494	0.9901	0.1815
sec_mut_2 (18)	*sec_E10* (52)	14	2.7370	0.2601	0.0036
sec_mut_3 (21)	*sec_E10* (52)	14	0.2562	0.8501	0.4151
sec_mut_4 (40)	*sec_E10* (52)	14	2.6110	0.8001	0.0057
sec_mut_5 (97)	*sec_E10* (52)	14	2.4743	0.0701	0.0081
sec_mut_6 (47)	*sec_E10* (52)	14	2.3644	0.9501	0.0151
sec_mut_7 (19)	*sec_E10* (52)	14	2.4586	0.1901	0.0075

1 To maintain the tablewise error rate for the 19 tests, we used the sequential Bonferroni test.

Supplementary Table 2. Results of ANOVA and Dunnet's test for changes in the number of trichomes between unmodified and mutated *E10* constructs.

ANOVA Table *D. melanogaster* dorsal	**SS**	**df**	**MS**
Treatment (between columns)	52952	8	6619
Residual (within columns)	13807	81	170.5
Total	66759	89	

Dunnett's Multiple Comparison Test	**Mean Diff.**	**q**	**P value**
mel_E10 vs *mel_mut_1*	-1.900	0.3254	n.s.
mel_E10 vs *mel_mut_2*	24.30	4.162	<0.001
mel_E10 vs *mel_mut_3*	18.70	3.203	<0.05
mel_E10 vs *mel_mut_4*	-5.800	0.9934	n.s.
mel_E10 vs *mel_mut_5*	-5.600	0.9591	n.s.
mel_E10 vs *mel_mut_6*	-13.40	2.295	n.s.
mel_E10 vs *mel_mut_7*	-2.200	0.3768	n.s.
mel_E10 vs *mel_mut_All*	69.90	11.97	<0.001

ANOVA Table *D. melanogaster* lateral	**SS**	**df**	**MS**
Treatment (between columns)	6950	8	868.7
Residual (within columns)	3662	81	45.21
Total	10612	89	

Dunnett's Multiple Comparison Test	**Mean Diff.**	**q**	**P value**
mel_E10 vs *mel_mut_1*	1.800	0.5986	n.s.
mel_E10 vs *mel_mut_2*	13.40	4.456	<0.001
mel_E10 vs *mel_mut_3*	13.30	4.423	<0.001
mel_E10 vs *mel_mut_4*	11.00	3.658	<0.01
mel_E10 vs *mel_mut_5*	15.60	5.188	<0.001
mel_E10 vs *mel_mut_6*	-0.2000	0.06651	n.s.
mel_E10 vs *mel_mut_7*	-3.300	1.097	n.s.
mel_E10 vs *mel_mut_All*	24.60	8.181	<0.001

ANOVA Table *D. sechellia* dorsal	**SS**	**df**	**MS**
Treatment (between columns)	20512	8	2564
Residual (within columns)	2370	81	29.26
Total	22882	89	

Dunnett's Multiple Comparison Test	**Mean Diff.**	**q**	**P value**
sec_E10 vs *sec_mut_1*	0.3000	0.1240	n.s.
sec_E10 vs *sec_mut_2*	-13.30	5.498	<0.001
sec_E10 vs *sec_mut_3*	-8.600	3.555	<0.01
sec_E10 vs *sec_mut_4*	-2.100	0.8681	n.s.
sec_E10 vs *sec_mut_5*	-4.600	1.901	n.s.
sec_E10 vs *sec_mut_6*	-3.800	1.571	n.s.
sec_E10 vs *sec_mut_7*	-0.4000	0.1653	n.s.
sec_E10 vs *sec_mut_All*	-50.20	20.75	<0.001

Supplementary Table 3. Effect of mutations of the *E10* construct. [(Mean number of trichomes rescued by mutated *E10* construct - Mean number of trichomes rescued by parental species *E10* construct) / (mean number of trichomes rescued by *mel_E10* construct - mean number of trichomes rescued by *sech_E10* construct)]*100. The mutations with significant effects are marked in bold.

D. melanogaster **dorsal+lateral**	
mel_mut_1	0.1
mel_mut_2	**-33.5**
mel_mut_3	**-28.5**
mel_mut_4	**-4.6**
mel_mut_5	**-8.9**
mel_mut_6	12.1
mel_mut_7	4.9
mel_mut_All	**-84.1**

D. sechellia dorsal+lateral	
sec_mut_1	1.0
sec_mut_2	**14.6**
sec_mut_3	**9.9**
sec_mut_4	3.4
sec_mut_5	5.9
sec_mut_6	5.1
sec_mut_7	1.7
sec_mut_All	**51.5**

Supplementary Material References

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