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Kyoto University
The gain of cis-regulatory activities underlies novel domains of wingless gene expression in Drosophila.

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Abstract

Changes in gene expression during animal development are largely responsible for the evolution of morphological diversity. However, the genetic and molecular mechanisms responsible for the origins of new gene expression domains have been difficult to elucidate. Here, we sought to identify molecular events underlying the origins of three novel features of wingless (\textit{wg}) gene expression that are associated with distinct pigmentation patterns in \textit{Drosophila guttifera}. We compared the activity of cis-regulatory sequences (enhancers) across the \textit{wg} locus in \textit{D. guttifera} and \textit{D. melanogaster} and found strong functional conservation among the enhancers that control similar patterns of \textit{wg} expression in larval imaginal discs that are essential for appendage development. For pupal tissues, however, we found three novel \textit{wg} enhancer activities in \textit{D. guttifera} associated with novel domains of \textit{wg} expression, including two enhancers located surprisingly far away in an intron of the distant \textit{Wnt10} gene. Detailed analysis of one enhancer (the vein-tip enhancer) revealed that it overlapped with a region controlling \textit{wg} expression in wing crossveins (crossvein enhancer) in \textit{D. guttifera} and other species. Our results indicate that one novel domain of \textit{wg} expression in \textit{D. guttifera} wings evolved by co-opting pre-existing regulatory sequences governing gene activity in the developing wing. We suggest that the modification of existing enhancers is a common path to the evolution of new gene expression domains and enhancers.
Introduction

As animals have adapted to diverse habitats, they have evolved many new and different kinds of body parts. One of the major outstanding questions in evolutionary biology is: what kinds of mechanisms underlie the origin of morphological novelties? It is well established that the regulatory genes responsible for the formation and patterning of animal bodies and body parts, the so-called “toolkit” genes for animal development, are shared and highly conserved among most animal phyla. (1-4). The fact that very different forms are generated by similar sets of developmental genes, and a large body of empirical, comparative studies, have led to the general consensus that divergence in the expression and regulation of toolkit genes and the genes they control largely underlies morphological diversity (5-9).

Similarly, several studies have revealed that new features of regulatory gene expression are associated with the evolution of morphological novelties, such as new color pattern elements on insect wings (10-14). How new patterns of regulatory gene expression evolve, however, has been more difficult to elucidate. In principle, new patterns of gene expression may evolve through: i) changes in the deployment of upstream trans-acting regulatory factors; ii) changes in the cis-regulatory sequences of the genes themselves; or iii) a combination of these mechanisms. For example, the novel, male-specific wing spot in *Drosophila biarmipes* and a few close relatives evolved through a combination of changes in the spatial expression of the trans-acting Distal-less (Dll) transcription factor and the evolution of Dll and other binding sites in a cis-regulatory element of at least one pigmentation gene (15, 16). In this case, the Dll protein is said to have been co-opted in the evolution of a new morphological trait.

However, the mechanism underlying the co-option of Dll is not known in this case, nor for any other instances of the co-option of regulatory genes. It is not known, for instance, whether new features of gene expression evolve via the de novo origin of enhancers, or through the transposition or modification of existing enhancers. One distinguishing feature shared by most developmental regulatory gene loci is that, like *Dll* (17, 18), they often contain vast cis-regulatory regions harboring numerous independent enhancers. To complicate matters, some of these enhancers may be located far away in other genes. The diversity of enhancers belonging to individual regulatory genes is explicit evidence that gene function has expanded in the course of evolution by accumulating additional enhancers, but understanding how this occurs presents significant experimental challenges. To further our understanding of the molecular basis of gene expression novelties, it is necessary both to identify the novel enhancers in the species of interest, and to ascertain their structural and functional relationships to sequences in other species lacking.
the specific domains of gene expression (19).

The Wg protein is a secreted signaling molecule that acts as a morphogen in the development of numerous structures and pattern elements in Drosophila and other animals. (20-23). Here, we have traced the molecular basis of three novel features of wingless (wg) gene expression in Drosophila guttifera that are associated with three distinct features of adult pigmentation. By searching through the wg and adjacent loci of both D. guttifera and D. melanogaster, we found three novel enhancer activities in D. guttifera. We show that one of these enhancers, the novel vein-tip enhancer in D. guttifera, is nestled within a conserved enhancer in other species. We propose that the new enhancer activity evolved through the modification of the preexisting enhancer.

Results

Novel wg expression domains in the D. guttifera pupal wing

Regulatory genes coordinate important developmental events, thus their expression patterns are constrained and usually conserved, particularly among closely related species. wg expression patterns in larval imaginal discs (wing disc, eye-antennal disc, and leg disc) of D. melanogaster and D. guttifera adhere to this generality and are essentially identical (Fig. S1). In both species, wg expression were virtually identical in the developing wing pouches and the future nota of wing discs (Figs. S1A and S1D), the anterior-ventral parts of antennae, ventral and dorsal sides of eye discs (Figs. S1B and S1E), and anterior-ventral parts of leg discs (Figs. S1C and S1F).

In contrast, in the developing pupal wings of Drosophila guttifera, wg is expressed in two domains that are not present in D. melanogaster pupal wings (14, Fig. 1). Whereas in D. melanogaster, wg is expressed in cells along the developing wing margin (henceforth "margin") and crossveins (“crossveins”, Fig. 1A, arrows), in D. guttifera (Fig. 1B) wg is also expressed at the tips of longitudinal veins ("vein tip", asterisks) and in precursors of the campaniform sensilla ("campaniform sensilla", arrowheads). None of the other several species closely related to D. guttifera within the D. quinaria species group (D. deflecta, D. nigromaculata, D. palustris and D. quinaria) exhibited wg expression in the developing campaniform sensilla or vein-tips (Fig. S2). Both novel wg expression domains correlate with color pattern formation in D. guttifera (Fig. 1D), but not in D. melanogaster (Fig. 1C).

The wg enhancers active in imaginal discs are conserved between species
Our primary task was to identify the enhancers responsible for these novel features of \( \text{wg} \) expression in \( D. \text{guttifera} \). Because we could not predict where novel enhancers might be located, our approach was to identify functional enhancers across the entire \( D. \text{guttifera} \) \( \text{wg} \) region and to compare their activity and structure with the homologous regions of the \( D. \text{melanogaster} \) \( \text{wg} \) region. This approach offered the added benefit of enabling a comparison of the overall organization of the cis-regulatory regions of the \( \text{wg} \) locus of the two species. Because we could not assume that \( D. \text{guttifera} \) enhancers would have the same activity in \( D. \text{melanogaster} \) (the usual host for transgenic methods in Drosophila) as in \( D. \text{guttifera} \), we constructed reporter genes with DNA from each species and injected them into their species of origin. The \( D. \text{melanogaster} \) \( \text{wg} \) locus sequence had been determined previously (20, 22, 24). From a draft assembly of the \( D. \text{guttifera} \) genome and PCR amplification, we located the \( D. \text{guttifera} \) \( \text{wg} \) locus on a 64 kb long contig. We systematically fused 0.3-10kb (average 4.0kb) non-coding segments of each species’ \( \text{wg} \) region to an EGFP/DSRed reporter gene (Fig. S3).

We first monitored the larval imaginal discs for reporter protein activity, and we were able to confirm or identify several orthologous \( \text{wg} \) enhancers (Fig. 2) including: i) a previously reported enhancer driving wing pouch expression in the 5’ region in both species (Figs 2B and 2E; the \( \text{spade}^{\text{dog}} \) \((\text{spdfg})\) region in \( D. \text{melanogaster} \); 25); ii) enhancers active in the eye-antennal discs and leg discs that are located in the 3’ region of \( \text{wg} \) gene (Figs 2C and 2F); and iii) an enhancer in the 3’ region of the \( \text{Wnt}6 \) gene (Figs. 2D and 2G). These results are consistent with a recent survey describing a large collection of imaginal disc enhancers of \( D. \text{melanogaster} \) (Flylight; 26). Because the \( \text{Wnt}6 \) expression pattern is mostly similar to that of \( \text{wg} \) (27), and the clustering of four \( \text{Wnt} \) genes is conserved in the \( Drosophila \) genus, the loci have been inferred to share regulatory elements (28). We note that the overall position and order of imaginal disc enhancers is largely colinear across the 60-74 kb region in both species (Fig. 2A). This result indicates that there have not been any significant inversions or other rearrangements across the region in either lineage since the two species diverged from a common ancestor approximately 63 million years ago (the divergence between the subgenus \( Drosophila \) and subgenus \( \text{Sophophora} \), 29).

**The \( D. \text{guttifera} \) \( \text{wg} \) locus contains a novel vein-tip enhancer**

In our search for enhancers that regulate the \( D. \text{guttifera} \)-specific \( \text{wg} \) expression domains in pupal wings, we identified two enhancers located 3’ of the \( D. \text{guttifera} \) \( \text{wg} \) gene: a crossveins enhancer (\( \text{gutCV-T} \)) and margin enhancer (\( \text{gutME} \)), which together account for the conserved \( \text{wg} \)-expression domains (Figs. 3A-C; see also Fig. 1B). We also identified the
orthologous enhancers, melCV and melME, from *D. melanogaster*, which drove reporter expression in the crossveins and wing margin, respectively (Fig. 3D and 3E). Importantly, in addition to the conserved crossvein expression, gutCV-T also drove reporter expression in the developing wing tips, where the wing veins meet the margin, which is part of the novel *wg* expression pattern in *D. guttifera* (Fig. 3B).

The difference in activities between the orthologous melCV and gutCV-T enhancers of *D. melanogaster* and *D. guttifera* could be due to differences in trans-acting regulatory factors expressed in each wing, differences in cis-regulatory sequences between the enhancers, or both. To determine which might be the case, we carried out a simple cis-trans test by introducing the gutCV-T enhancer into *D. melanogaster*. The gutCV-T fragment drove reporter protein expression in both the crossveins and vein-tips in pupal *D. melanogaster* wings (Fig. 3F). This result indicates that the trans-acting factors necessary for the vein-tip expression pattern are present in both species. Thus, the differences in activities between the gutCV-T and melCV enhancers must reside in their cis-regulatory sequences.

We also isolated and tested the orthologous cis-regulatory region from *D. deflecta*, which is one of the most closely related species to *D. guttifera* but does not have *wg* expression in the vein tips (Fig. S2C). This *D. deflecta* crossveins enhancer (defCV) drove reporter protein expression in the wing crossveins in *D. guttifera*, but showed no activity in the vein tips (Fig. 3G). This result indicates that the vein-tip enhancer activity is unique to *D. guttifera*, and that the novel feature of *wg* expression in *D. guttifera* wing vein tips arose through the evolution of cis-regulatory sequences in the *D. guttifera* lineage, after it split off from a common ancestor shared with *D. deflecta*.

**The *D. guttifera*-specific vein-tip enhancer is nestled within the crossvein enhancer**

The 2.4 kb gutCV-T enhancer, which drove both crossvein and vein-tip expression in *D. guttifera* (Fig. 3B), shares numerous collinear, highly-conserved blocks of sequence with both the 1.8 kb melCV fragment from the *D. melanogaster* locus and the 1.7 kb defCV fragment from the *D. deflecta* locus, which both lack vein-tip activity (Fig. 3D and 3G; Fig. 4A). We considered two possibilities to explain how the novel vein tip expression of gutCV-T may have evolved within the domain of the crossvein enhancer: i) a distinct enhancer element, able to independently drive expression in the vein tips, inserted into the *D. guttifera* *wg* locus (by chance next to another pupal wing enhancer); or ii) a novel activity arose within the crossvein enhancer that utilized and is dependent upon pre-existing sites in the crossvein enhancer. To attempt to distinguish these
possibilities, we compared the *D. guttifera*, *D. deflecta*, and *D. melanogaster* sequences and searched for major insertions or regions unique to *D. guttifera*. Indeed, we found that the *D. guttifera* fragment is over 900 bp longer than the orthologous *D. melanogaster* sequence and 600 bp longer than the orthologous *D. deflecta* sequence (Fig. 4A). This size difference is largely due to a region in the less conserved 5’ end of the gutCV-T enhancer. This additional sequence did not show any similarity to known transposable elements (when tested by blastn against the NCBI nucleotide collection). To test whether this region might contain a distinct enhancer, we divided gutCV-T into two fragments; the insert-containing 5’ 1653bp (gutCVT5) and the 3’, highly-conserved 756bp fragment (gutCVT-core) (Fig. 4A). While the gutCVT5 fragment showed no activity, the gutCVT-core fragment drove expression in both the crossoveins and vein tips (Figs. 4B and 4C). These results reveal that the novel activity in the gutCVT enhancer arose within the 3’ 756bp region.

To examine how this region may have acquired its unique vein tip activity, we compared it in detail with the orthologous *D. deflecta* sequence that lacks vein tip activity. The *D. guttifera* CVT-core region is 83% similar to the orthologous *D. deflecta* region, with many large blocks of identical sequence and just a few small (<10bp) insertions or deletions (Fig. S4). This pattern of sequence homology indicates that the novel domain of wg expression in the vein tips of *D. guttifera* is likely due to a small number of nucleotide changes and/or small indels nestled within the well-conserved crossovein enhancer.

**The campaniform sensillum and thoracic stripe enhancers are in the distant Wnt10 region**

During our initial search for wg enhancer activities in *D. guttifera*, we were puzzled by our inability to find an enhancer for the novel patterns of wg expression in the developing wing campaniform sensilla, which contributes several spots to the overall polka-dotted wing pattern (14). Therefore, we expanded our search into adjacent *Wnt* loci just in case they might contain enhancers that regulate wg transcription. Using seven additional scaffolds, we extended the region analyzed to include a 174 kb region containing the *Wnt4*, *Wnt6*, and *Wnt10* genes. We were surprised to find two more distinct enhancer activities in the *Wnt10* region, more than 69kb away from the wg transcription start site and separated from it by the *Wnt6* locus (Fig. 5A; Fig. S3; see also Fig. 2). One 5kb fragment within the second intron of the *Wnt10* gene (gutCS; Fig. 5A) drove reporter expression in the campaniform sensilla and along the anterior margin of the pupal wing (Fig. 5B, arrowheads). Because wg is the only gene in this *Wnt* cluster that is expressed in campaniform sensilla (Fig. S5), we conclude that this enhancer controls wg
expression.

A second, partially overlapping 4.3 kb fragment (gutTS, Fig. 5A) drove reporter expression in a series of thoracic stripes (Fig. 5C) that correspond well with the adult thoracic striped pigmentation pattern (Fig. 5D). We were not able to confirm by in situ hybridization that this reflects a native wg expression domain because gene probes did not yield reliable signals in pupal thoracic body wall tissues. However, we performed RT-PCR on thoracic body wall total RNA to ascertain which Wnt genes were active in this tissue. Only wg showed strong expression while the other, adjacent Wnt genes (Wnt4, Wnt6, and Wnt10) exhibited weak or no expression (Fig. S6). These results, and the strong correlation with thoracic pigmentation, indicate that wg is expressed in the thorax and regulated by the gutTS enhancer.

**Cis-regulatory sequence evolution is partly responsible for novel distant enhancer activities**

We next sought to identify the relative contribution of cis-acting and trans-acting regulatory factors in the evolution of the *D. guttifera* gutCS and gutTS enhancer activities. We conducted reciprocal tests of the *D. guttifera* and homologous *D. melanogaster* sequence in the other species’ genetic background. Contrary to the gutCV-T enhancer, the *D. guttifera* CS enhancer was not active in *D. melanogaster* wings, indicating a role for trans-acting factors in enhancer activity in *D. guttifera* (Fig. S7C). In addition, the homologous *D. melanogaster* fragment (45.7% similarity) was not active in either *D. guttifera* or *D. melanogaster*, indicating an additional contribution of cis-regulatory changes in the gutCS enhancer (Fig. S7A and S7B). Taken together, these results indicate that both cis-regulatory and trans-regulatory changes were responsible for the evolution of the novel wg expression domain in campaniform sensilla.

We performed a similar set of reciprocal experiments with the gutTS enhancer and homologous *D. melanogaster* sequence (Fig. S8). The homologous fragment from *D. melanogaster* (46.3% similarity) was inactive in both *D. melanogaster* (Fig. S8A) and *D. guttifera* (Fig. S8B), whereas the *D. guttifera* TS enhancer was weakly active in stripes in the *D. melanogaster* thorax (compare Fig. S8C and Fig. S8D). These results indicate that cis-regulatory changes are largely responsible for the novel activity of the gutTS enhancer and that some, but perhaps not all, of the trans-acting factors involved in regulating the enhancer are deployed in *D. melanogaster*.

**Discussion**

A large body of comparative studies has shown that changes in the spatiotemporal
expression of toolkit genes and their target genes they regulate correlate with the evolution of morphological traits. In a considerable number of instances, these spatiotemporal changes in gene expression have been demonstrated to involve the modification of enhancers (6, 7, 30-36). However, there are relatively few cases in which the origins of new enhancers have been elucidated, and none involving regulatory genes themselves.

Here, we have shown that three novel domains of \( \text{wg} \) expression in \( D. \text{guttifera} \) are governed by three novel enhancers, respectively (Fig. 6). We found that the evolution of \( \text{wg} \) cis-regulatory sequences within the \( D. \text{guttifera} \) lineage played a role in the gain of each enhancer activity, and that the evolution of trans-acting regulatory factors was also necessary for the activity of two elements (gutCS and gutTS). Detailed analysis of the \( D. \text{guttifera} \) vein-tip enhancer revealed that it evolved within another conserved enhancer, while two other enhancers (the campaniform sensilla and thoracic stripe enhancers) arose within in an intron of the distant \( \text{Wnt10} \) locus. These results bear on our understanding of the mechanisms underlying the evolution of new enhancers and domains of gene expression.

The origin of the vein-tip enhancer via co-option of an existing enhancer

The \( D. \text{guttifera} \) vein-tip enhancer activity was localized within a 756 bp DNA segment that was also active in the developing pupal crossveins. This DNA segment is orthologous to segments of DNA in \( D. \text{melanogaster} \) and \( D. \text{deflecta} \) that were only active in the crossveins. The segments are all collinear, and contain numerous blocks of identical sequence, which suggests that the vein tip enhancer activity evolved within the pre-existing crossvein enhancer. This inference is further supported by the observation that we were unable to separate the two enhancer activities by subdivision of the 756bp fragment.

One explanation for the presence of two inseparable activities in this one fragment is that they share functional sites – i.e., binding sites for common transcription factors. Because both activities appear in the pupal wing, it is likely that they utilize common tissue-specific (wing) and temporal (pupal) inputs. The evolution of a new activity in the vein tips could have arisen through the addition of DNA-binding sites for TFs that were already present active in cells at vein tips. In this scenario, the novel enhancer activity would have resulted from the evolutionary co-option of an existing enhancer.

There is precedent for multifunctional enhancers and for this mechanism of co-option. For example, one enhancer of the \( D. \text{melanogaster \ even-skipped} \) gene governs two domains of gene expression that are controlled by shared inputs (37). In addition, Rebeiz et al. (19)
demonstrated that a novel optic lobe enhancer of the *Drosophila santomea Neprilysin-1* gene arose via co-option of an existing enhancer. Moreover, it was shown that co-option had occurred in just a few mutational steps. The co-option of existing elements is an attractive explanation for the evolution of novel enhancers because it requires a relatively short mutational path.

**The evolution of distant cis-regulatory elements**

One surprising property of enhancers is their ability to control gene transcription at promoters located at considerable linear distances away in the genome (38-40). For example, the enhancer that drives *Sonic hedgehog* (*Shh*) expression in the developing amniote limb bud is located in the intron of another gene approximately 1 megabase (Mb) from the *Shh* locus (41, 42). A growing body of evidence indicates that long segments of DNA are looped out in accommodating long-range enhancer-promoter interactions (43, 44). The ability of enhancers to act over such long ranges suggests that new enhancers could evolve at considerable distances from the promoters that they regulate.

Here, we identified two enhancers in an intron of the *D. guttifera Wnt10* gene that control transcription of the *wg* gene from a distance of ~70 kb, and separated by the *Wnt6* locus. Our data suggests that the gutTS enhancer preferentially regulates *wg* transcription and not *Wnt10* or *Wnt6* transcription, although we cannot offer any explanation at present for this preference. The origins of the gutCS and gutTS enhancers are not as clear as the vein tip enhancer. We did not detect any pupal enhancer activity in the orthologous DNA segments of *D. melanogaster*, so we do not have any evidence of enhancer co-option. Nor did we find any obvious insertions in these DNA segments such as a transposon. Nevertheless, the discovery of these novel, distant elements reflects the functional flexibility of cis-regulatory elements and their contribution to the evolution of gene regulation and morphological diversity.

**Materials and Methods**

**Fly strains and genomic DNA**

*Drosophila melanogaster* Canton-S (wild-type) was used for genomic DNA preparation and expression analysis of *Wnt* genes. We obtained *D. guttifera* (stock no. 15130-1971.10), *D. deflecta* (15130-2018.00), *D. quinaria* (15130-2011.00), and *D. palustris* (15130-2001.00) from the Drosophila Species Stock Center at University of California, San Diego, and *D. nigromaculata* (strain no. E-14201) from EHIME-Fly, Ehime University, Japan.
Genomic DNA was extracted and purified using a squish method (45) and Genomic tip-20/G columns (Qiagen, Hilden, Germany).

**In situ hybridization**

Species specific, partial sequences of Wnt genes (Wnt4, wg, Wnt6 and Wnt10) were amplified by PCR from genomic DNA and cloned into the pGEM-TEasy vector (Promega, Madison, WI). PCR products re-amplified from the plasmid clones were in vitro transcribed to produce DIG-RNA probes (35). Imaginal discs of late 3rd instar larvae and wings of P6 stage pupae (46) were subjected to in situ hybridization as described previously (14, 47). Specimens were mounted and imaged under a stereomicroscope SZX-16 (Olympus, Tokyo).

**Genomic sequence of the Wnt locus**

The genome sequence reads of D. guttifera were obtained with a Genome Analyzer IIx (Illumina, San Diego, CA), and assembled with CLC workbench (CLC Bio, Aarhus, Denmark). The Wnt locus of D. guttifera was reconstructed with seven genomic scaffolds and genomic PCR products (Accession no. KP966547, Fig. S3). For the comparison of sequences from multiple species, we used GenePalette software (48). All primers are listed in Table S1.

**EGFP/DsRed reporter assay for enhancer activity using transgenic Drosophila**

For the site-specific integration of transgenes into D. melanogaster, the plasmid vector S3aG (36), fly strains VK00006 (cytogenetic location 19E7)(49) and ZH-attP-51D (cytogenetic location 51D)(50) were used. D. guttifera transgensics were made according to the previously described method (14), using the cloning shuttle vector pSLfa1180fa harboring DsRed2 or DsRed.T4, the piggyBac transposon vector pBac\{3xP3-EGFPafm\}(51, 52) and the piggyBac helper plasmid phspBac (53). Fluorescent reporter expression was observed under a stereomicroscope SZX-16 and a confocal laser-scanning microscope FV1000 (Olympus, Tokyo).

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Author contributions

The authors have made the following declarations about their contributions: Conceived and designed the experiments: SK MWG TW SBC. Performed the experiments: SK MWG KV VAK JHY TW. Analyzed the data: SK MWG VAK SBC. Contributed reagents/materials/analysis tools: SK MWG KV VAK JHY TW SBC. Contributed to the writing of the manuscript: SK MWG TW SBC.

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References


Figure Legends.

Figure 1. Unique wingless expression domains in Drosophila guttifera pupal wings correlate with adult pigment spots. (A) wg expression pattern in the pupal wing of D. melanogaster visualized by in situ hybridization. wg is expressed in the developing crossveins and along the wing margin. (B) wg expression pattern in the pupal wing of D. guttifera. wg is expressed in the campaniform sensilla (arrowheads), crossveins (arrows) and longitudinal vein tips (asterisks), and along the entire wing margin. (C) Adult wing of D. melanogaster. (D) Adult wing of D. guttifera.

Figure 2. Conserved wg cis-regulatory elements control similar gene expression patterns in Drosophila imaginal discs. (A) Schematic of enhancers plotted on the wg locus of D. melanogaster and D. guttifera. Solid vertical lines connected by horizontal gray lines represent sequences longer than 40bp with 100% nucleotide conservation between species. (B-D) D. melanogaster third instar imaginal discs showing reporter expression with D. melanogaster enhancer fragments (EGFP, green). (E-G) D. guttifera third instar imaginal discs showing very similar reporter expression patterns driven by orthologous D. guttifera enhancer fragments (DsRed, magenta). All discs are oriented with anterior to the left and dorsal on top. w: wing disc. ea: eye-antennal disc. l: leg disc. (Magnification: B–G, 200x.)

Figure 3. A novel vein tip enhancer activity in D. guttifera. (A) Schematic of pupal wing enhancers in D. guttifera and D. melanogaster. Black bars connected by gray lines represent sequences longer than 40bp with 100% nucleotide conservation between species. Inset: Schematic of wg expression in the pupal wing that is color-coded for the responsible enhancers. (B) D. guttifera pupal wing showing reporter expression from the gutCV-T enhancer in the crossveins and vein tips (DsRed, magenta). (C) D. guttifera pupal wing showing reporter expression from the gutME enhancer (DsRed, magenta) along the wing margin. (D) D. melanogaster pupal wing showing reporter expression from the melCV enhancer fragment (EGFP, green) in the crossvein. (E) D. melanogaster pupal wing showing reporter expression from the melME enhancer (EGFP, green) along the wing margin. (F) D. melanogaster pupal wing showing reporter expression from the gutCV-T enhancer (EGFP, green) in the crossveins and vein tips. (G) D. guttifera pupal wing showing reporter expression (DsRed, magenta) from the defCV enhancer in the crossveins (asterisks). (Magnification: B–G, 100x)
Figure 4. The *D. guttifera* vein-tip enhancer is nestled within a conserved crossvein enhancer. 

(A) Schematic comparing crossvein enhancer regions in *D. melanogaster*, *D. guttifera*, and *D. deflecta*. The gutCV-T enhancer (gray bar) aligned with the melCV enhancer (black bar, top) and the defCV enhancer (blue bar, bottom) using GenePalette (gray boxes connected with gray lines indicate sequences of 15 bp or longer with 100% conservation between species) and Vista Browser (50bp sliding window with percent sequence identity indicated, peaks with greater than 80% sequence identity are shaded in pink). Peaks show extent of sequence conservation in a sliding 50bp window. The gutCV-T enhancer was divided into two fragments, gutCVT5 (yellow bar) and gutCVT-core (green bar). 

(B) *D. melanogaster* pupal wing showing absence of reporter expression from gutCVT5 (EGFP, green). 

(C) *D. melanogaster* pupal wing showing reporter expression from the gutCVT-core fragment (EGFP, green) in the crossveins (arrows) and vein tips (asterisks). (Magnification: B and C, 100x.)

Figure 5. The distant *Wnt10* region contains two novel and distinct *wg* enhancers in *D. guttifera*. 

(A) Schematic showing the location of two enhancer fragments in the second intron of *Wnt10*. 

(B) *D. guttifera* pupal wing showing reporter expression driven by the gutCS enhancer (DsRed, magenta) in the campaniform sensilla (arrowheads). 

(C) *D. guttifera* pupal thorax showing a striped reporter expression pattern driven by the gutTS enhancer (DsRed, magenta). 

(D) Stripes of black pigmentation on the thorax of an adult *D. guttifera*. (Magnification: B, 80x.; C, 50x.; D, 32x.)

Figure 6. Three novel *wg* enhancers drive *D. guttifera*-specific pigmentation patterns. The genomic organization of the *D. guttifera* *Wnt* region is shown with colored shapes corresponding to enhancers from this study. The pupal expression domains of each enhancer are mapped by their respective color onto the pigmentation patterns of the adult animal.

Figure S1. *wg* expression patterns in imaginal discs are conserved between two *Drosophila* species. *In situ* hybridizations with third instar larval imaginal discs. 

(A) *D. melanogaster* wing disc. 

(B) *D. melanogaster* eye-antennal disc. 

(C) *D. melanogaster* leg disc. 

(D) *D. guttifera* wing disc. 

(E) *D. guttifera* eye-antennal disc. 

(F) *D. guttifera* leg disc. All discs are oriented with anterior to the left and dorsal on top.
**Figure S2.** wg expression in longitudinal vein tips and campaniform sensilla of the pupal wing is unique to *D. guttifera*. *In situ* hybridization for the *wg* gene is shown in pupal wings of various species. (A) *Drosophila melanogaster*. (B) *D. guttifera*. (C) *D. deflecta*. (D) *D. nigromaculata*. (E) *D. palustris*. (F) *D. quinaria*. *D. melanogaster* belongs to *melanogaster* species-group of the subgenus *Sophophora*, while the other species belong to the *quinaria* species group of the subgenus *Drosophila*. All probes are species-specific.

**Figure S3.** Map of the Wnt region of *D. melanogaster* and *D. guttifera*. Vertical bars connected with black lines indicate sequences of 40bp or longer with 100% sequence conservation between species. Red lines indicate conserved but inverted sequences. Numbered horizontal solid bars indicate DNA fragments tested by transgenic reporter assays. *D. melanogaster* fragments were tested in transgenic *D. melanogaster* using phiC31 integration. *D. guttifera* fragments were tested in transgenic *D. guttifera* using the *piggyBac* transposon.

**Figure S4.** The novel activity of the *D. guttifera* gutCV-T enhancer arose within a conserved enhancer. Sequence alignment of the gutCV-T enhancer and the orthologous defCV enhancer (Serial Cloner v2.6.1, local alignment, and word size=15 were used). Mismatches are marked with a #. Red bars indicate identical sequence matches >10bp with the melCV-core enhancer from *D. melanogaster*. The extent of collinear sequence conservation (with no significant rearrangements) between the two species indicates that a small change or an accumulation of small changes is responsible for the new enhancer activity in the vein tips.

**Figure S5.** Expression patterns of four *Wnt* genes in pupal wings of *D. guttifera* visualised by *in situ* hybridizations. (A) Wnt4. (B) wingless. (C) Wnt6. (D) Wnt10.

**Figure S6.** *wingless* is the predominant *Wnt* gene expressed in the pupal thorax. *Wnt* genes expressed in the pupal thorax (top) and embryo (middle) were detected by RT-PCR and reaction products profiled by gel electrophoresis. Control reactions from genomic DNA are shown at the bottom.

**Figure S7.** Cis- and trans-regulatory changes are responsible for the novel campaniform sensillum expression of *wg* in *D. guttifera*. (A) In the *D. melanogaster* pupal wing, the melCS enhancer shows no restricted expression (EGFP, green). (B) In the *D. guttifera* pupal wing, the
melCS enhancer shows no expression (DsRed, magenta). (C) *D. melanogaster* pupal wing, the gutCS enhancer shows no restricted expression (EGFP, green). (E) *D. guttifera* pupal wing, the gutCS enhancer drives reporter expression in the campaniform sensillum (DsRed, magenta).

**Figure S8.** *Cis*- and *trans*-regulatory changes are responsible for the striped expression of *wg* in the pupal thorax of *D. guttifera*. (A) *D. melanogaster* pupal thorax, the melTS fragment shows no stripe expression (EGFP, green). (B) *D. guttifera* pupal thorax, the melTS fragment shows no stripe expression (DsRed, Magenta). (C) *D. melanogaster* pupal thorax, the guts enhancer is expressed in incomplete stripes (EGFP, green). (D) *D. guttifera* pupal thorax, the gutTS enhancer drives full stripe expression which corresponds to the adult pigmentation pattern (DsRed, magenta).
Figure 2

**D. melanogaster**

- B
- C
- D

**D. guttifera**

- E
- F
- G

Images show expression patterns of genes in the indicated species.

- ** wg**: Wingless
- ** Wnt6**: Wnt-6

The figure illustrates genomic localization and transcriptional activity of these genes.
Figure 3
Figure 4

A

B

C

melanogaster
gutCVT5

melanogaster
gutCVT-core
Figure 5

(A) Schematic representation of the Wnt10 gene region showing the gutCS and gutTS transcription start sites. The scale bar represents 1 kb.

(B) Image showing the expression pattern of gutCS and gutTS. White arrows indicate specific expression sites.

(C) Detailed view of the gutTS region, highlighting the expression pattern.

(D) Anatomical view of the region of interest.
Figure S1

D. melanogaster

A

B

C

D. guttifera

D

E

F

Figure S1
Figure S2

A. *D. melanogaster*

B. *D. guttifera*

C. *D. deflecta*

D. *D. nigromaculata*

E. *D. palustris*

F. *D. quinaria*
**Figure. S3**

D. melanogaster sequences tested in D. melanogaster

D. guttifera sequences tested in D. guttifera

D. melanogaster sequences tested in D. melanogaster

D. guttifera sequences tested in D. guttifera

10kb
Figure S4
Embryo cDNA

Pupa thorax cDNA

Genomic DNA

Figure. S6
Figure. S8

*D. melanogaster*

A. melTS

B. melTS

C. gutTS

D. gutTS

*D. guttifera*

A. melTS

B. melTS

C. gutTS

D. gutTS
Table S1. Primers used in the study.

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Note: The table lists primers used in the study, their restriction sites, and the fragments they target, along with the host species and template species for each primer set.
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ACT\textasciitilde T\textasciitilde TG\textasciitilde GC\textasciitilde C\textasciitilde GC\textasciitilde C\textasciitilde G\textasciitilde C\textasciitilde G\textasciitilde TA\textasciitilde AT\textasciitilde AT\textasciitilde GA\textasciitilde G\textasciitilde C\textasciitilde A\textasciitilde T\textasciitilde A\textasciitilde A\textasciitilde AAA\textasciitilde AT\textasciitilde T\textasciitilde T\textasciitilde G\textasciitilde A\textasciitilde SbfI 10

melanogaster melanogaster S3aG

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melanogaster melanogaster S3aG

TCT\textasciitilde TT\textasciitilde G\textasciitilde GC\textasciitilde G\textasciitilde C\textasciitilde C\textasciitilde A\textasciitilde T\textasciitilde A\textasciitilde AT\textasciitilde G\textasciitilde T\textasciitilde G\textasciitilde AA\textasciitilde C\textasciitilde AC\textasciitilde GT\textasciitilde TAGT\textasciitilde AscI 12

melanogaster melanogaster S3aG

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melanogaster melanogaster S3aG

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melanogaster melanogaster S3aG

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melanogaster melanogaster S3aG

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"Primers for cis/trans test (Figure 3, 4, 5, S7, S8)"

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