Gain of *cis*-regulatory activities underlies novel domains of *wingless* gene expression in *Drosophila*

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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* (*wg*) gene expression that are associated with distinct pigmentation patterns in *Drosophila guttifera*. We compared the activity of *cis*-regulatory sequences (enhancers) across the *wg* locus in *D. guttifera* and *Drosophila melanogaster* and found strong functional conservation among the enhancers that control similar patterns of *wg* expression in larval imaginal discs that are essential for appendage development. For pupal tissues, however, we found three novel *wg* enhancer activities in *D. guttifera* associated with novel domains of *wg* expression, including two enhancers located surprisingly far away in an intron of the distant *Wnt10* gene. Detailed analysis of one enhancer (the vein-tip enhancer) revealed that it overlapped with a region controlling *wg* expression in wing crossveins (crossvein enhancer) in *D. guttifera* and other species. Our results indicate that one novel domain of *wg* expression in *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.

enhancers | novelty | gene regulation | development | pigmentation

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). Very different forms are generated by similar sets of developmental genes, and a large body of empirical, comparative studies has 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 example, 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 Wingless (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 *Drosophila 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 pre-existing 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 (*SI Appendix*, Fig. S1). In both species, *wg* expression was virtually identical in the developing wing pouches and the future nota of wing discs (*SI Appendix*, Fig. S1 *A* and *D*), the anterior-ventral parts of antennae, ventral and dorsal sides of eye discs (*SI Appendix*, Fig. S1 *B* and *E*), and the anterior-ventral parts of leg discs (*SI Appendix*, Fig. S1 *C* and *F*).

In contrast, in the developing pupal wings of *D. 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 (Fig. 1*A*, arrows), in *D. guttifera* (Fig. 1*B*) *wg* is also expressed at the tips of longitudinal veins ("vein tip") (Fig. 1*B*, asterisks) and in precursors of the campaniform sensilla (Fig. 1*B*, arrowheads). None of the other several species closely related to *D. guttifera* within the *Drosophila quinaria* species group (*Drosophila deflecta*, *Drosophila nigromaculata*, *Drosophila palustris*, and *D. quinaria*) exhibited *wg* expression in the developing campaniform sensilla or vein tips (*SI Appendix*, Fig. S2). Both novel *wg* expression domains correlate with color pattern formation in *D. guttifera* (Fig. 1*D*), but not in *D. melanogaster* (Fig. 1*C*).

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 *wg* expression in *D. guttifera*. Because we could not predict where novel enhancers might be located, our approach was to identify functional enhancers across the entire *D. guttifera wg* region and to compare their activity and structure with the homologous regions of the *D. melanogaster wg* region. This approach offered the added benefit of enabling a
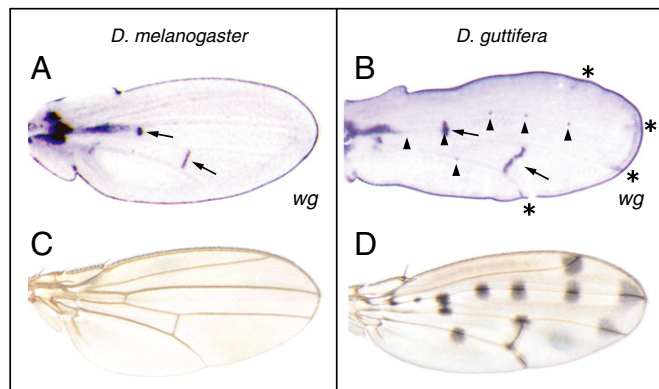

Fig. 1. Unique *wg* expression domains in *D. guttifera* pupal wings correlate with adult pigment spots. (*A*) *wg* expression pattern in the pupal wing of *D. melanogaster* visualized by in situ hybridization. *wg* is expressed in the developing crossveins and along the wing margin. (*B*) *wg* expression 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*.
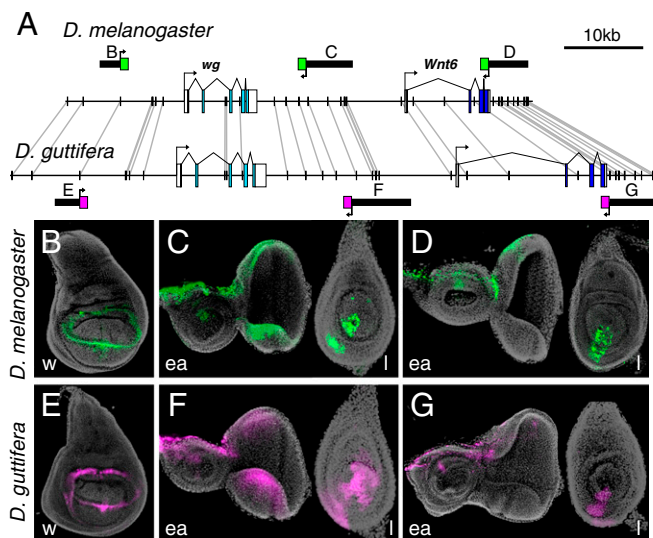

Fig. 2. Conserved *wg cis*-regulatory elements control similar gene-expression patterns in *Drosophila* imaginal discs. (*A*) Schematic of enhancers plotted on the *wg* locus of *D. melanogaster* and *D. guttifera*. Solid vertical lines connected by gray lines represent sequences longer than 40 bp with 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. ea, eye-antennal disc; l, leg disc; w, wing disc. (Magnification: *B–G*, 200×.)

comparison of the overall organization of the *cis*-regulatory regions of the *wg* locus of the two species. Because we could not assume that *D. guttifera* enhancers would have the same activity in *D. melanogaster* (the usual host for transgenic methods in *Drosophila*) as in *D. guttifera*, we constructed reporter genes with DNA from each species and injected them into their species of origin. The *D. melanogaster wg* locus sequence had been determined previously (20, 22, 24). From a draft assembly of the *D. guttifera* genome and PCR amplification, we located the *D. guttifera wg* locus on a 64-kb-long contig. We systematically fused 0.3- to 10-kb (average 4.0 kb) noncoding segments of each species' *wg* region to an EGFP/DsRed reporter gene (*SI Appendix*, Fig. S3).

We first monitored the larval imaginal discs for reporter protein activity, and we were able to confirm or identify several orthologous *wg* enhancers (Fig. 2) including: (*i*) a previously reported enhancer driving wing pouch expression in the 5′ region in both species (Fig. 2 *B* and *E*) [the *spade*^flag^ (*spd*^fg^) region in *D. melanogaster* (25)]; (*ii*) enhancers active in the eye-antennal discs and leg discs that are located in the 3′ region of *wg* gene (Fig. 2 *C* and *F*); and (*iii*) an enhancer in the 3′ region of the *Wnt6* gene (Fig. 2 *D* and *G*). These results are consistent with a recent survey describing a large collection of imaginal disc enhancers of *D. melanogaster* (Flylight) (26). Because the *Wnt6* expression pattern is mostly similar to that of *wg* (27), and the clustering of four 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- to 74-kb region in both species (Fig. 2*A*). 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 ~63 million y ago (the divergence between the subgenus *Drosophila* and subgenus *Sophophora*) (29).

The *D. guttifera wg* Locus Contains a Novel Vein-Tip Enhancer. In our search for enhancers that regulate the *D. guttifera*-specific *wg* expression domains in pupal wings, we identified two enhancers

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located 3′ of the *D. guttifera wg* gene: a crossveins enhancer (gutCV-T) and margin enhancer (gutME), which together account for the conserved *wg*-expression domains (Fig. 3 *A–C*; see also Fig. 1*B*). We also identified the orthologous enhancers, melCV and melME, from *D. melanogaster*, which drove reporter expression in the crossveins and wing margin, respectively (Fig. 3 *D* and *E*). 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. 3*B*).

The difference in activities between the orthologous melCV and gutCV-T enhancers of *D. melanogaster* and *D. guttifera* could be because of 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. 3*F*). 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 (*SI Appendix*, Fig. S2*C*). 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. 3*G*). 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. 3*B*), 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 (Figs. 3 *D* and *G* and 4*A*). 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 used 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. 4*A*). This size difference is largely because of 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 National Center for Biotechnology Information nucleotide collection). To test whether this region might contain a distinct enhancer, we divided gutCV-T into two fragments: the insert-containing 5′ 1,653-bp (gutCVT5) and the 3′, highly conserved 756-bp fragment (gutCVT-core) (Fig. 4*A*). Whereas the gutCVT5 fragment showed no activity, the gutCVT-core fragment drove expression in both the crossveins and vein tips (Fig. 4 *B* and *C*). These results reveal that the novel activity in the gutCVT enhancer arose within the 3′ 756-bp 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 (<10 bp) insertions or deletions (*SI Appendix*, Fig. S4). This pattern of sequence homology indicates that the novel domain of *wg* expression in the vein tips of *D. guttifera* is likely caused by a small number of nucleotide changes or small indels nestled within the well-conserved crossvein 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-dot 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
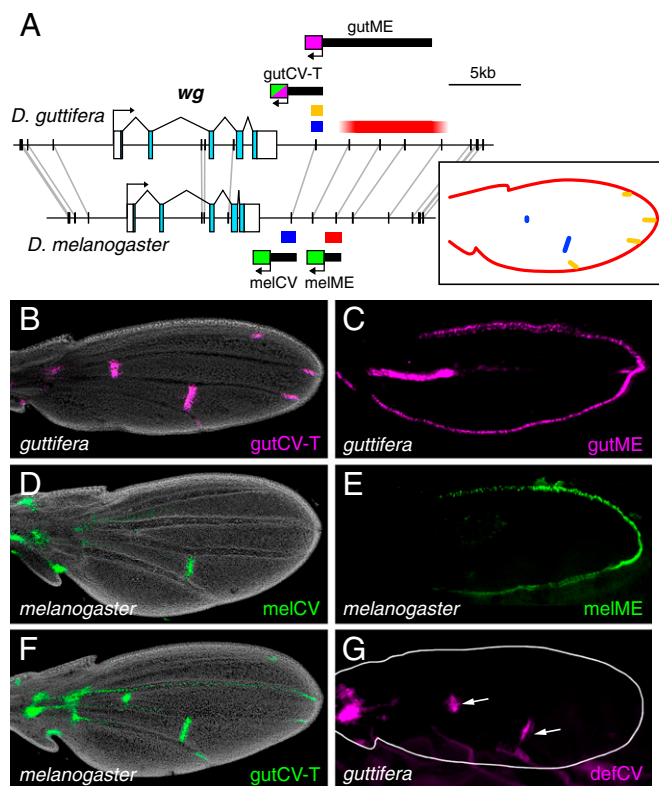

Fig. 3. A novel vein-tip enhancer activity in *D. guttifera*. (*A*) Schematic of pupal wing enhancers in *D. guttifera* and *D. melanogaster*. Black bars connected by gray lines represent sequences longer than 40 bp with 100% nucleotide conservation between species. (*Inset*) Schematic of *wg* expression 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*, 100×.)
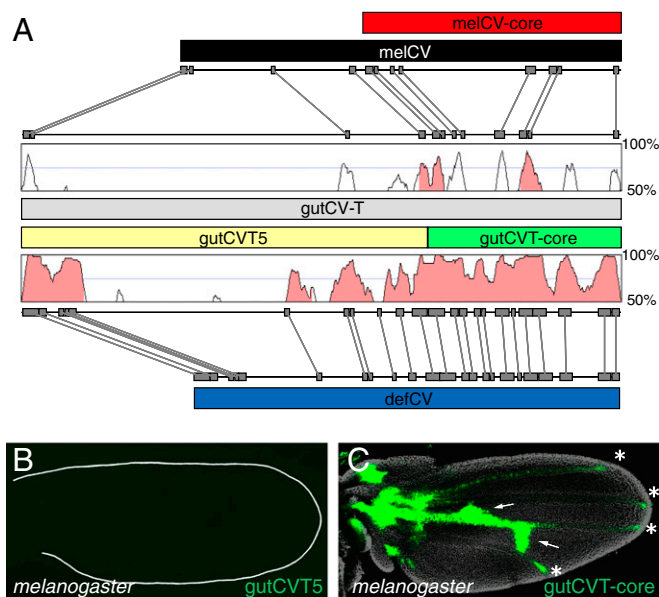
Fig. 4. The *D. guttifera* vein-tip enhancer is nestled within a conserved crossvein enhancer. (*A*) Schematic comparing crossvein enhancer regions in *D. melanogaster*, *D. guttifera*, and *D. deflecta*. The gutCV-T enhancer (gray bar) aligned with the melCV enhancer (black bar, *Top*) and the defCV 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 (50-bp 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 50-bp 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*, 100×.)

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 69 kb away from the *wg* transcription start site and separated from it by the *Wnt6* locus (Figs. 2 and 5*A*, and *SI Appendix*, Fig. S3). One 5-kb fragment within the second intron of the *Wnt10* gene (gutCS) (Fig. 5*A*) drove reporter expression in the campaniform sensilla and along the anterior margin of the pupal wing (Fig. 5*B*, arrowheads). Because *wg* is the only gene in this *Wnt* cluster that is expressed in campaniform sensilla (*SI Appendix*, Fig. S5), we conclude that this enhancer controls *wg* expression.

A second, partially overlapping 4.3-kb fragment (gutTS) (Fig. 5*A*) drove reporter expression in a series of thoracic stripes (Fig. 5*C*) that correspond well with the adult thoracic striped pigmentation pattern (Fig. 5*D*). 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, whereas the other, adjacent *Wnt* genes (*Wnt4*, *Wnt6*, and *Wnt10*) exhibited weak or no expression (*SI Appendix*, 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. In contrast 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* (*SI Appendix*, 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 (*SI Appendix*, Fig. S7 *A* and *B*). 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 (*SI Appendix*, Fig. S8). The homologous fragment from *D. melanogaster* (46.3% similarity) was inactive in both *D. melanogaster* (*SI Appendix*, Fig. S8A) and *D. guttifera* (*SI Appendix*, Fig. S8B), whereas the *D. guttifera* TS enhancer was weakly active in stripes in the *D. melanogaster* thorax (compare *SI Appendix*, Fig. S8 *C* and *D*). 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*.
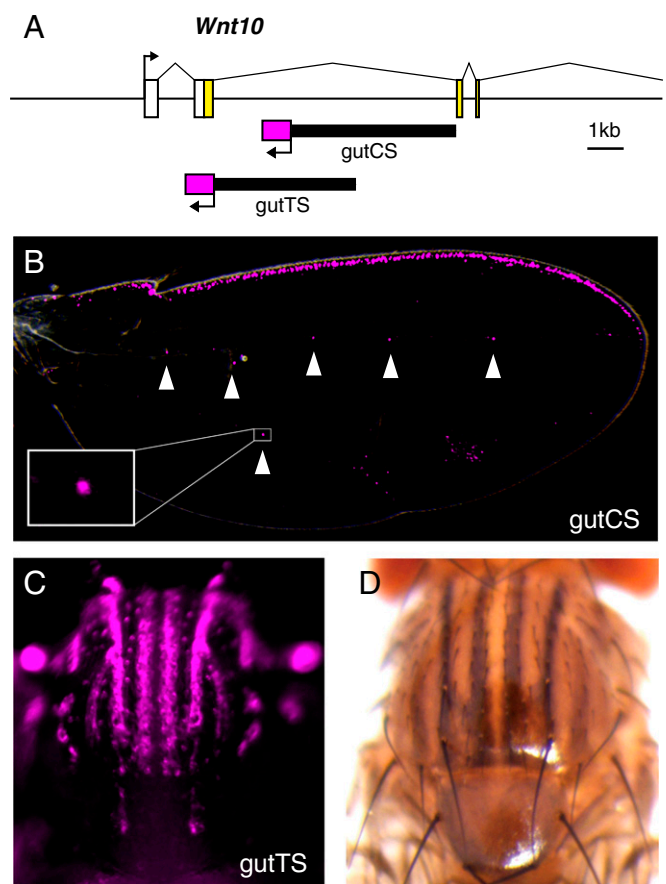

Fig. 5. The distant *Wnt10* region contains two novel and distinct *wg* enhancers in *D. guttifera*. (*A*) Schematic showing the location of two enhancer fragments in the second intron of *Wnt10*. (*B*) *D. guttifera* pupal wing showing reporter expression driven by the gutCS enhancer (DsRed, magenta) 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*, 80×; *C*, 50×; *D*, 32×.)

Discussion

A large body of comparative studies has shown that changes in the spatiotemporal expression of toolkit genes and the 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 *wg* expression in *D. guttifera* are governed by three novel enhancers, respectively (Fig. 6). We found that the evolution of *wg cis*-regulatory sequences within the *D. 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. guttifera* vein-tip enhancer revealed that it evolved within another conserved enhancer, whereas two other enhancers (the campaniform sensilla and thoracic stripe enhancers) arose within in an intron of the distant *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. 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. melanogaster* and *D. 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.

One explanation for the presence of two activities in this one fragment is that they share functional sites: that is, binding sites for common transcription factors. Because both activities appear in the pupal wing, it is likely that they use 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 transcription factors 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. 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 ~1 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 suggest 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.
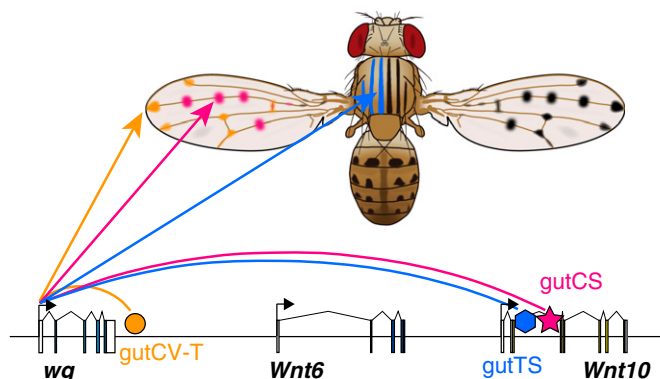
Materials and Methods

Fig. 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.

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EVOLUTION