Chance caught on the wing: *cis*-regulatory evolution and the origin of pigment patterns in *Drosophila*

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The gain, loss or modification of morphological traits is generally associated with changes in gene regulation during development. However, the molecular bases underlying these evolutionary changes have remained elusive. Here we identify one of the molecular mechanisms that contributes to the evolutionary gain of a male-specific wing pigmentation spot in *Drosophila biarmipes*, a species closely related to *Drosophila melanogaster*. We show that the evolution of this spot involved modifications of an ancestral *cis*-regulatory element of the *yellow* pigmentation gene. This element has gained multiple binding sites for transcription factors that are deeply conserved components of the regulatory landscape controlling wing development, including the selector protein Engrailed. The evolutionary stability of components of regulatory landscapes, which can be co-opted by chance mutations in *cis*-regulatory elements, might explain the repeated evolution of similar morphological patterns, such as wing pigmentation patterns in flies.

The evolution of new morphological features is due predominantly to modifications of spatial patterns of gene expression. Changes in the expression of a particular gene can result from alterations either in its *cis*-regulatory sequences or in the deployment and function of the *trans*-acting transcription factors that control it, or both. Understanding the evolution of new morphological traits thus requires both the identification of genes that control trait formation and the elucidation of the *cis*- and *trans*-modifications that account for gene expression differences.

Evolution of *cis*-regulatory elements has been proposed to be a major source of morphological diversification because mutations in regulatory elements can produce discrete tissue-specific expression pattern changes while avoiding deleterious pleiotropic effects[1–3]. In the best-studied cases of gene expression changes underlying morphological divergence, *cis*-regulatory modifications have been proposed[4–6], occasionally suggested by genetic evidence[7–10], but have only rarely been formally demonstrated[11] or analysed at the molecular level[12,13]. It is currently not known whether the evolution of new morphological traits occurs largely through the modification of pre-existing *cis*-regulatory elements or from the generation of new elements; neither is it understood how many or what kinds of modifications are required for a regulatory element to drive a novel pattern.

To address these issues, we have analysed the evolution of a conspicuous male-specific wing pigmentation pattern in *Drosophila biarmipes*, a species closely related to *Drosophila melanogaster*[14] (Fig. 1). Wing pigmentation patterns in insects are highly diversified and have various biological functions including mimicry, camouflage, thermoregulation, and mate selection[15]. In *D. biarmipes*, the sexually dimorphic wing pattern is associated with a courtship behaviour in which males display their wings conspicuously to the females, suggesting a function for this spot in mate choice[16,17]. This wing spot has evolved recently in some species of the *D. melanogaster* group, such as *D. biarmipes*, and it is absent from close outgroup species such as *D. pseudoobscura*[17] (Fig. 1).

Formation of wing pigmentation results from the conversion of melanin precursors diffusing from the veins into pigment deposits at specific positions along the wing, wherever converting proteins are present[18]. The product of the *yellow* (*y*) gene is required for the production of black pigments, and the distribution of its product prefigures adult pigmentation patterns[11,19]. The Yellow protein is expressed uniformly at low levels throughout the developing wings of *D. melanogaster* and *D. pseudoobscura*, where it imparts a low overall level of melanic pigmentation. In contrast, in *D. biarmipes*, in addition to the low, uniform expression, Yellow protein is highly expressed in an anterior distal spot[19] (Fig. 1). This tight correlation between a novel Yellow expression pattern and a novel pigmentation

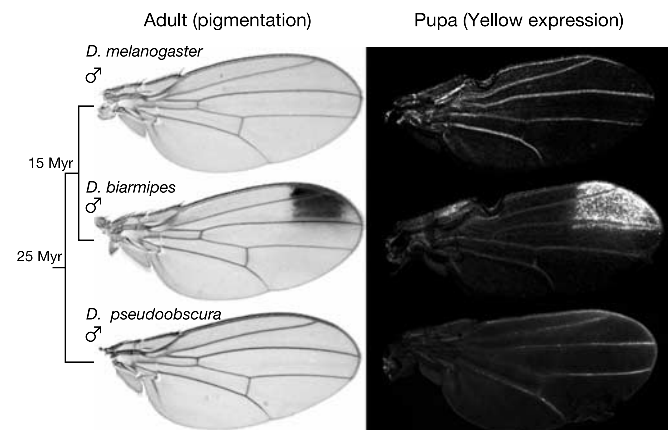

Figure 1 Expression of the Yellow protein prefigures adult wing pigmentation. The conspicuous spot of dark pigmentation present at the tip of the male wing of *Drosophila biarmipes* (left) is a new trait evolved among species of the *Drosophila melanogaster* group[14,46] (about 15 Myr of divergence; divergence time is 60–80 Myr for the family Drosophilidae[30]), superimposed on the ancestral pattern of uniform grey shading and darker veins found both in *D. melanogaster* and in *D. pseudoobscura*, a species from the sister *D. obscura* group (25 Myr of divergence[29,30]). In all three species the male pupal distribution of Yellow in the wing, revealed by a specific antibody (right), foreshadows the adult pigmentation.

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pattern prompted us to ask whether regulatory evolution at the *y* locus underlies the novel distribution of the Yellow protein in *D. biarmipes*, or whether this is due to changes in *trans*-acting regulators of *y*.

Regulatory changes in *cis* to the *yellow* locus

To test whether the observed differences in Yellow expression between *D. biarmipes* and *D. melanogaster* (Fig. 1) are due to changes at the *y* locus, we transformed *D. melanogaster* with green fluorescent protein (GFP)-reporter constructs containing non-coding DNA from the *D. biarmipes y* (y^{bia}) locus. If relevant evolutionary changes have occurred in *cis*, then the reporter gene might be regulated in *D. melanogaster* in a manner similar to the native *y* gene in *D. biarmipes*. If, however, the changes have occurred in *trans*, the *D. biarmipes y* regulatory element might drive reporter expression similar to that of the *y* gene in *D. melanogaster* (that is, uniformly). We found that *D. melanogaster* transgenic flies carrying the entire 5′ region (8 kilobases; Fig. 2a) of the y^{bia} gene ($5'$ y^{bia}) express GFP in the pupal wings in a pattern similar to the native *D. biarmipes* Yellow expression (Fig. 2b). Low levels of GFP are uniformly distributed across the wing, and higher levels of GFP are confined to the distal part of the anterior compartment. This result shows that the transcription factors deployed in the developing wing of *D. melanogaster* recognize y^{bia} *cis*-regulatory sequences. Furthermore, the *D. biarmipes*-like expression pattern in a *D. melanogaster trans*-regulatory context shows that evolutionary changes in Yellow expression involve primarily *cis*-regulatory modifications at the *y* locus, which presumably entail the gain (or loss) of binding sites for transcription factors.

The $5'$ y^{bia} element does not recapitulate the precise restriction of the native spot of Yellow expression; higher levels of reporter protein expression extend along the proximal–distal axis, indicating that additional regulatory differences exist between *D. biarmipes* and *D. melanogaster*. Additional reporter constructs suggest that these differences are *trans* effects or are due to *cis*-acting elements located outside the region we have tested. The unique intron of the *D. biarmipes y* gene does contain another *cis*-regulatory element (for all developing sensory bristles) but has no activity in the wing other than in these sense organs. Furthermore, a transgene containing the 5′ non-coding, 5′ untranslated region, first exon, intron and second exon sequences (partial locus, Fig. 2a) is expressed in a similar pattern to that of the $5'$ y^{bia} element, indicating that the differences in *y* expression are not due to any of these sequences (data not shown).

Having localized major regulatory differences to the y^{bia} 5′ region, we next investigated whether the novel *cis*-regulatory activity of the y^{bia} region arose in a pre-existing regulatory element or evolved *de novo* in the *D. biarmipes* lineage.

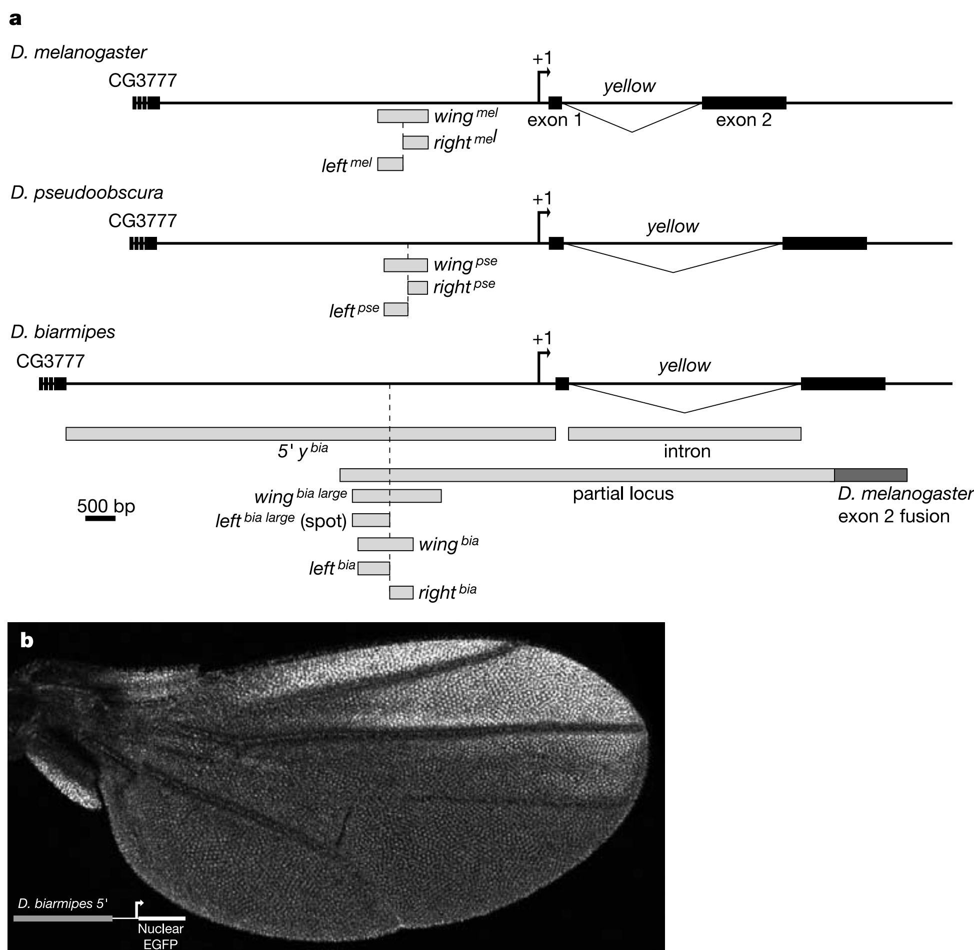

Figure 2 *Cis*-regulatory changes at the *yellow* locus are responsible for species-specific differences in Yellow distribution. **a**, The organization of the *y* locus is similar in *Drosophila melanogaster*, *Drosophila biarmipes* and *D. pseudoobscura*. **b**, The entire 5′ region of *D. biarmipes y*, comprising sequences between the coding sequences of *y* and the closest predicted gene (CG3777), is sufficient to drive reporter GFP expression in *D. melanogaster* at a time and in a pattern similar to those of *y* expression in native *D. biarmipes*. The y^{bia} intron does not drive wing expression other than in the marginal sensory bristles, and the partial locus drives expression in a pattern similar to the entire 5′ region of y^{bia} (not shown). Black boxes, coding sequence; grey boxes, fragments analysed in transgenic constructs.

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Evolution of a wing-specific *cis*-regulatory element

In *D. melanogaster*, analysis of the *y* regulatory region has revealed that an 800-base-pair (bp) element located 1 kilobase upstream of the transcription start site, named the *wing^{mel}* element (Fig. 2a), is sufficient to drive gene expression throughout the pupal wing (Fig. 3b) and is necessary for adult wing pigmentation[11,20,21]. We hypothesized that functional modifications of this element might account for differences in Yellow expression between the wings of *D. melanogaster* and *D. biarmipes*. There is strong sequence conservation of this portion of the *y* locus between the two species (Fig. 3a and Supplementary Fig. 1). We transformed *D. melanogaster* with a GFP-reporter construct containing a 920-bp fragment from *D. biarmipes* orthologous to the *D. melanogaster wing* element (*wing^{mel}*), termed *wing^{bia}* (Fig. 2a). This fragment drives a reporter pattern resembling that driven by the *5′ y^{bia}* element, with slightly less contrast between the levels of overall expression in the wing and in the anterior distal area (not shown). A larger fragment encompassing *wing^{bia}*, named *wing$^{bia\ large}$* (1,542 bp; Fig. 2a) drives a reporter pattern identical to the *5′ y^{bia}* element (Fig. 3b). These results indicate that the sequences required for the strong anterior-distal activation of Yellow expression in *D. biarmipes* pupal wings are located within and immediately adjacent to a wing-specific *cis*-regulatory element that is orthologous to the *wing^{mel}* element.

To determine whether the novel *wing^{bia}* sequences evolved within an ancestral *wing cis*-regulatory element, we examined *D. pseudoobscura*, an outgroup species that belongs to a clade generally devoid of wing pigmentation patterns other than the grey (light black) homogeneous shading (Fig. 1). Phylogenetic character reconstruction suggests that the pigmentation spot was present in the common ancestor of *D. biarmipes* and *D. melanogaster* and has been lost in the *D. melanogaster* lineage[22,23]. There is substantial sequence conservation at the *y* gene between *D. biarmipes* and *D. pseudoobscura* (Fig. 3a and Supplementary Fig. 1), which allowed us to identify a region in *D. pseudoobscura* that is orthologous to the *wing^{bia}* element, named *wing^{pse}* (724 bp). This *wing^{pse}* element drives ubiquitous wing expression (Fig. 3b), demonstrating that a functional *wing* element is ancestral to the *D. melanogaster*/*D. biarmipes* lineage and that sequences within and/or adjacent to this element were modified to control high levels of expression in the anterior distal part of the wing in *D. biarmipes*.

To understand the organization of the *wing^{bia}* element and to localize its novel functional sequences, we further dissected the *wing^{bia}* element. We found that the sequences necessary for the anterior distal expression are separable from those controlling the general wing expression in *D. biarmipes*. Two complementary, non-overlapping sequences of the *wing^{bia}* element, *right^{bia}* and *left^{bia}* (Fig. 2a), drive respectively ubiquitous expression throughout the wing blade and strong activation in the anterior distal area of the wing (Fig. 3b) (as do the complementary subfragments from the *wing$^{bia\ large}$* element, *right$^{bia\ large}$* (not shown) and *left$^{bia\ large}$*; Fig. 4b). A similar dissection clearly separates two wing-specific complementary functions in *D. pseudoobscura* (ubiquitous expression, and expression around the veins) but yields non-functional elements in *D. melanogaster* (Fig. 3b). These results indicate that sites in both regions of the *wing* element are required for its function in *D. melanogaster* and *D. pseudoobscura*, and that some or all of the novel sequences in *D. biarmipes* responsible for the specific anterior distal wing expression of Yellow are located in the *left$^{bia\ large}$* element, hereafter referred to as the *spot* element. The distinct and robust activities of the two parts of the *wing^{bia}* element raise the possibility that the *wing* element has been subfunctionalized in *D. biarmipes* into two elements controlling expression throughout the wing and in the spot, respectively.

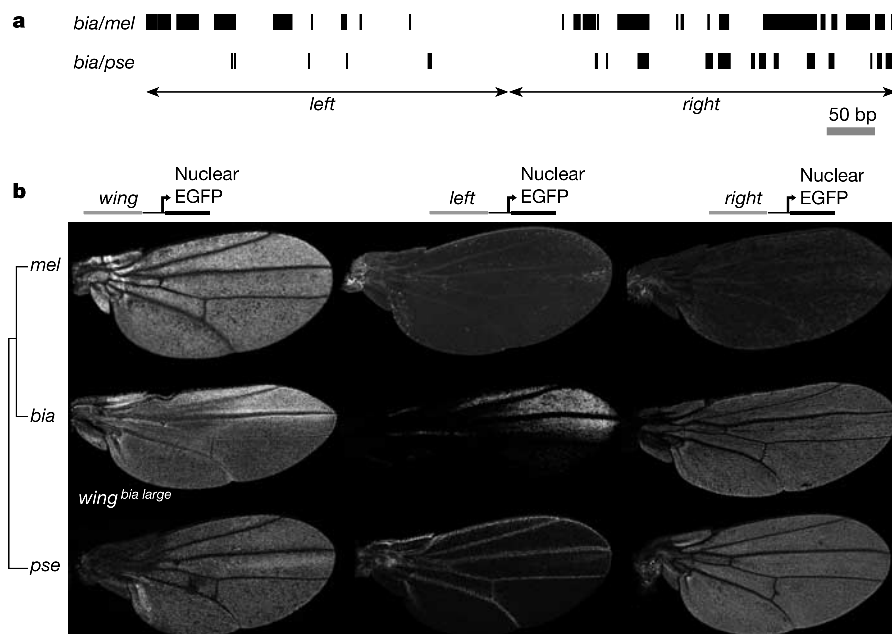

Figure 3 The *cis*-regulatory sequences governing spot formation evolved in the context of an ancestral wing enhancer. **a**, Conservation of the *wing* element sequence between *D. biarmipes* (*bia*) and *D. melanogaster* (*mel*) or *D. pseudoobscura* (*pse*) determined by Vista[47] with a 10-base-pair window length; only conservation above 75% is shown as solid boxes. Arrows show the boundaries of the *left* and *right* fragments. **b**, Reporter expression driven by the orthologous *wing* elements and its subfragments *left* and *right* (columns) of *D. melanogaster* (top), *D. biarmipes* (middle; the *wing$^{bia\ large}$* element is shown) and *D. pseudoobscura* (bottom), all expressed in *D. melanogaster*. The ubiquitous expression driven by the outgroup species *wing^{pse}* element (expression is present in vein cells at a lower levels comparable to those in *left^{pse}*) shows that the sequences responsible for the spot pattern in *D. biarmipes* have evolved in the context of an ancestral wing regulatory element. The sequences controlling the spot pattern are separable from those controlling general expression in *D. biarmipes* (*left* and *right*). Note that the posterior boundary of activity of the *left^{bia}* construct lies near or at the anterior–posterior compartment boundary.

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Multiple sites evolved in the wing spot element

In principle, the evolution of the spot pattern could arise through gaining binding sites for a single transcription factor that is expressed precisely in the cells that form the spot pattern. Alternatively, the spot pattern could result from the evolution of a combination of binding sites for multiple activators, as well as potential repressors that might restrict expression to this area. Resolution of these possibilities bears on the general question of the number of steps involved in the evolution of new patterns of gene expression and *cis*-regulatory element function.

To distinguish between these possibilities, we derived a series of reporter gene constructs with smaller portions of the 675-bp *spot* element. A 196-bp construct (*335–530*; Fig. 4a) retained activity in the anterior distal region of the wing, although we noted that reporter expression now extended into the posterior compartment (Fig. 4d). This suggested that one or more sites critical for activation

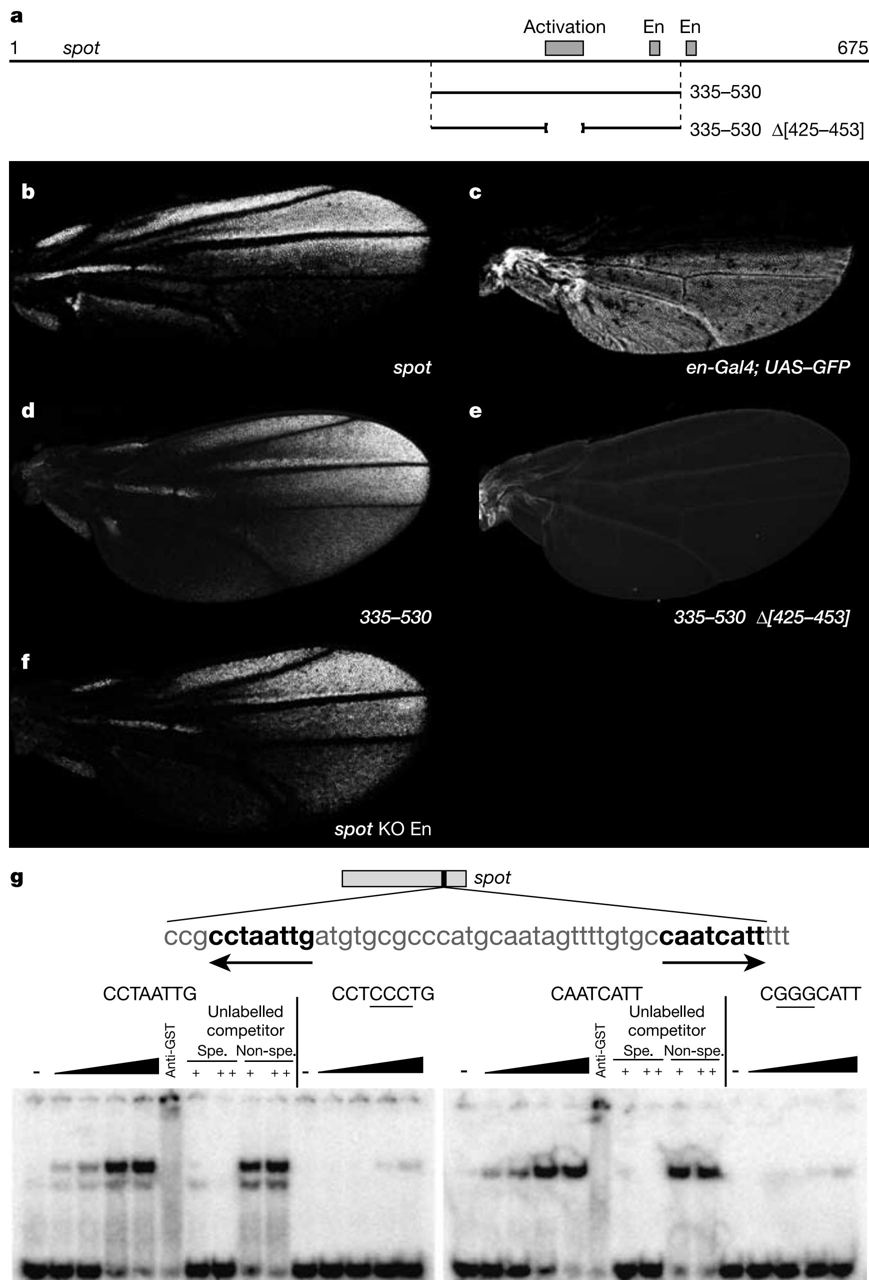

Figure 4 The *spot* element evolved through the acquisition of sites for both activators and repressors. **a**, Schematic of the 675-base-pair *spot* element showing the boundaries of deletion constructs and the location of identified binding sites. **b**, **d**–**f**, Expression of GFP driven by the *spot* element (**b**) and related constructs. **c**, The anterior border of expression of the selector gene *engrailed* abuts the *spot* element expression domain. **d**, A 196-base-pair element drives the *spot* pattern but is derepressed in the posterior compartment. **e**, Deletion of bp 425–453 abolishes activity of the *spot* element, indicating that sites required for activation lie within this region. **f**, Disruption of two characterized Engrailed binding sites from the *spot* element derepresses reporter expression in the posterior compartment. **g**, The two candidate sites are bound specifically by the Engrailed protein *in vitro*. Increasing amounts of Engrailed homeodomain–GST fusion protein (0.25–5 nM) specifically shift labelled DNA oligonucleotides representing native sequences containing putative binding sites (left part of each gel) but not sequences in which Engrailed sites have been mutated (underlined in the sequences, right part of each gel). Addition of anti-GST antibody supershifts complexes. Addition of specific (spe.) or non-specific (non-spe.) unlabelled competitor DNA (+, 50 ng; ++, 500 ng) reveals the specificity of the formation of complexes. Supershift and competition experiments were performed in the presence of 5.0 nM protein.

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resided in this 196-bp element and that one or more sites necessary for the restriction of expression from the posterior compartment resided outside it.

To localize further sequences required for activation of the *spot* element, we constructed a series of small deletions spanning the length of the 196-bp element (Fig. 4a, e, and data not shown). We found that a fragment lacking the internal sequences from bp 425 to 453 completely lacked reporter expression (Fig. 4e). This indicates that sequences required for activation in the spot are located within or overlap with bp 425–453. Together, these results indicate that sites for both at least one activator and one repressor have evolved in the *spot* element.

Direct regulation of the *spot* element by Engrailed

We next sought to identify potential *trans*-acting factors that regulate the *spot* element in *D. biarmipes*. The conspicuous posterior boundary of gene expression observed with the $left^{bia}$ and *spot* elements (Figs 3b and 4b) is reminiscent of the compartment boundary of the wing[24] defined by the anterior border of expression of the selector transcription factor Engrailed[25] (Fig. 4c). The posterior expansion of GFP expression in the deletion constructs shown in Fig. 4d would be consistent with posterior repression of the *spot* element by Engrailed.

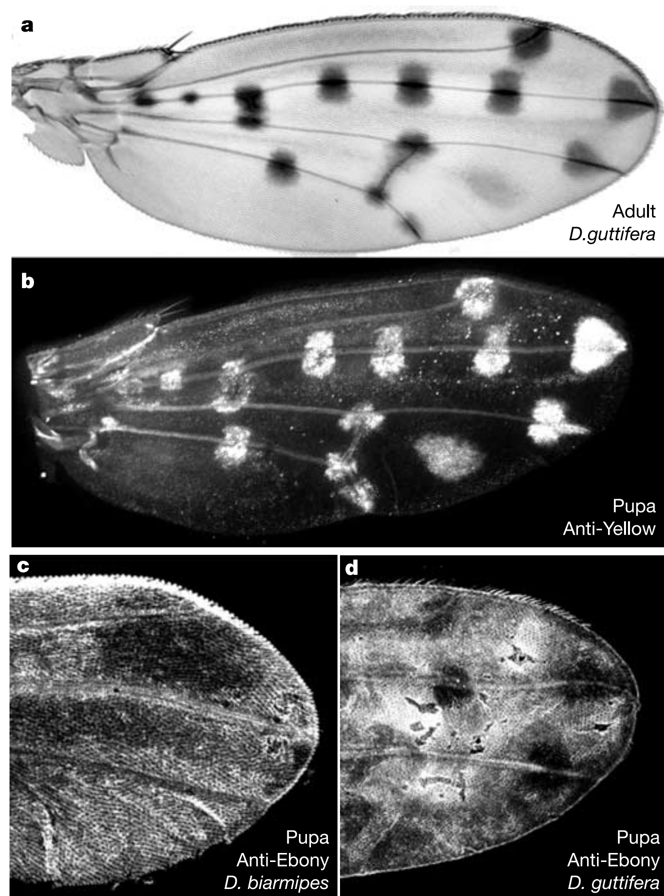

Figure 5 Concerted changes in the expression of Yellow and Ebony underlie the evolution of novel wing patterns. **a**, The distant species *D. guttifera* (a member of the *D. quinaria* group[48]) has evolved a complex pattern of dark spots located at the intersection of wing veins and where campaniform sensilla form. The grey shading is also reinforced in some interveins. **b**, The pupal distribution of Yellow also prefigures this adult pattern. **c**, In *Drosophila biarmipes*, the spatial repression of Ebony is also associated with the formation of the adult male spot of pigmentation[19]. **d**, This repression of Ebony associated with pigmentation patterns seems to be general, because it is also seen in *D. guttifera*, where the adult spots will form.

To test whether Engrailed might be a direct regulator of the $wing^{bia}$ element, we searched the *spot* sequence for putative Engrailed binding sites[26] and identified several candidate sites. Two of these sites, clustered within 43 bp, are specific to the *D. biarmipes spot* element (absent from *D. melanogaster* and *D. pseudoobscura* elements; Supplementary Fig. 1) and one site is located outside the 196-bp construct that exhibits some reporter expression in the posterior compartment (Fig. 4d). Gel-shift experiments on the native and mutated versions of these two sites showed that Engrailed binds specifically to them *in vitro* (Fig. 4g). Disruptions of these two Engrailed binding sites in the context of the *spot* element result in the specific derepression of reporter gene expression in the posterior compartment (Fig. 4f). These results show that the selector protein Engrailed directly represses the expression of the *y* gene in the posterior compartment of the wing and is one of the inputs that shapes the contours of the wing spot in *D. biarmipes*.

Multistep and multigenic evolution of the spot

Although multiple *cis*-regulatory modifications at the *y* locus have produced a profound evolutionary change in Yellow protein expression, it is important to ascertain whether changes in this one gene are sufficient for the evolution of the physical trait or whether additional evolutionary events are required. We have found that changes at *y* alone are not sufficient to create a pigmentation spot.

D. melanogaster y mutants carrying the *D. biarmipes y* gene (Fig. 2a, partial locus) recover only their species-specific pigment patterns; no wing spot is generated (not shown). Additional loci must therefore be involved.

The formation of pigment patterns is a multigenic process, and evolution at other pigmentation loci could also contribute to pattern evolution[18,27,28]. The male spot of *D. biarmipes* is also associated with the localized downregulation of the melanin-inhibiting product of the *ebony* (*e*) gene during wing development[19] (Fig. 5c), in a pattern that is approximately the inverse of Yellow expression. This suggests that, at least, both the repression of *e* and the activation of *y* are necessary for the formation of a dark spot. Consistent with this hypothesis is the observation that in *D. melanogaster e* mutants carrying the y^{bia} partial locus transgene (Fig. 2a), a slight darkening is observed specifically in the anterior area of the wing where *yellow* is strongly expressed (data not shown). However, this darkening is not comparable to the intense pigmentation spot of *D. biarmipes*. Changes in the expression of other pigmentation genes must also be involved. Furthermore, we have not been able to test whether changes in the *trans*-acting regulatory network of *D. biarmipes* might also contribute to the unique patterns of gene expression in the area of the wing spot. Taken together, these results indicate that the evolution of the novel pigmentation pattern of *D. biarmipes* required changes at multiple loci.

To determine whether the inverse regulation of expression of *y* and *e* is a general mechanism for the evolution of novel wing pigmentation patterns, we examined the expression of these proteins in *D. guttifera*, a species that separated from the *D. melanogaster* lineage about 40 Myr ago[29]. This species has independently evolved a strikingly different and more complex wing pigmentation pattern (Fig. 5a). We found that the pattern of expression of the two proteins also exhibits an inverse relationship with higher levels of Yellow (Fig. 5b) and lower levels of Ebony (Fig. 5d) in the pupal wing where the eventual adult pigmentation spots will form. This indicates that the evolution of both *y* and *e* expression is involved in the formation and evolution of novel wing pigmentation patterns in drosophilids.

Chance caught on the wing: novelty by co-option

In drosophilid flies, the shape of the wings and the pattern of

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venation have not changed much over 60–80 Myr of evolution[30,31]. Their development and patterning are largely understood in *D. melanogaster* and the regulatory proteins involved are conserved[32]. One such protein, the selector protein Engrailed, is a deeply conserved feature of the compartmental organization of arthropod segments and appendages. In the *Drosophila* wing, Engrailed is part of the regulatory circuit that sequentially organizes the patterning of the anterior–posterior axis[33]. Here we have shown that the activity of this transcription factor has been co-opted to control a feature of the novel wing pigmentation pattern in *D. biarmipes* through the evolution of specific binding sites within, or in the immediate vicinity of, a wing-specific regulatory element of the *y* gene. Because the expression driven by the *spot* element is also spatially modulated in *D. melanogaster*, this indicates that other conserved components of the wing *trans*-regulatory landscape (that is, one or more activators) have similarly been co-opted by the evolution of binding sites within the *y wing* element.

These findings suggest a general means by which novel expression patterns and characters can arise (Fig. 6a). Specifically, the random mutation of ancestral *cis*-regulatory elements (including point and insertional mutations) generates potential binding sites. If and when these sites can be recognized by transcription factors expressed in cells in which the ancestral element is active, the pattern or level of gene expression may be modified (Fig. 6a), in a manner similar to the mechanism of gene co-option demonstrated by the vertebrate crystallin genes[34]. The patterns of expression of the eligible transcription factors are initially cryptic with respect to the target gene or trait, but these cryptic 'prepatterns' are revealed once functional binding sites have evolved in target genes. In this sense, and in this example, evolution is precisely a matter, as Jacques Monod put it, of 'chance caught on the wing'[35,36].

This model has two specific implications for the evolution of novel wing patterns. First, it explains how the observed diversity of wing pigmentation patterns might result from combinations of the numerous transcription factors expressed in the wing. Each of these combinations might constitute a distinct prepattern for pigmentation genes such as *y* or *e*, provided that the corresponding binding sites evolve in the proper *cis*-regulatory context. For instance, some of the spots on the wing of *D. guttifera* surround the sensory organs located on the veins, which form at similar positions in most drosophilids. This raises the possibility that transcription factors involved in the positioning of these landmark organs have been co-opted to change *y* or *e* regulation in *D. guttifera*. Second, this model might explain the widespread repeated evolution of strikingly similar pigmentation patterns observed in distantly related species (for instance, pigmentation patterns similar to those studied here have evolved independently in other dipterans; Fig. 6b). The evolutionary stability of the *trans*-regulatory landscape in drosophilid wings, reflected by the strong conservation of the wing shape and venation pattern in the family, suggests that similar pigmentation patterns might arise in parallel through the repeated evolution of binding sites for the same transcription factors in *cis*-regulatory regions of pigmentation genes. □

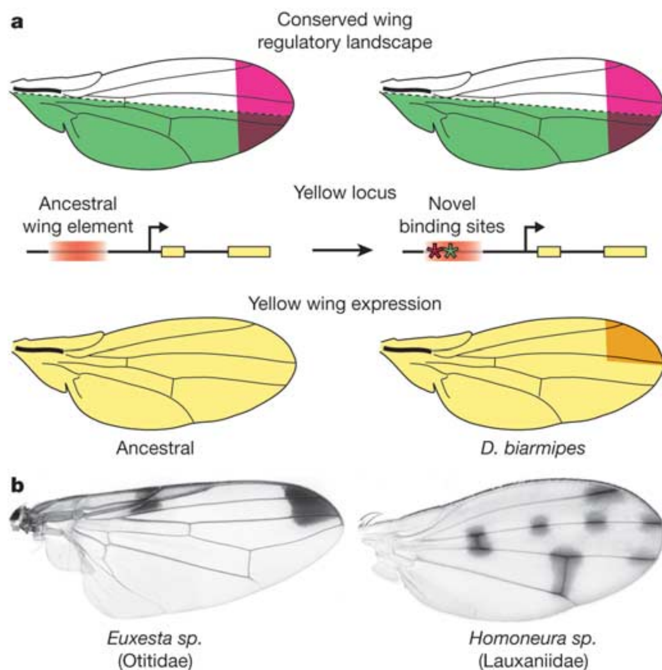

Figure 6 Cryptic prepatterns and the evolution of novel gene expression patterns through the evolution of *cis*-regulatory sequences. **a**, The upper panel shows a model of the conserved landscape of transcriptional regulators that pattern and shape the *Drosophila* wing (green and pink represent repressor and activator, respectively). The evolution of binding sites for a subset of these regulators in the *yellow* wing *cis*-regulatory element (coloured stars) co-opts them to modify *yellow* expression (lower panel). Combined with other regulatory changes at other loci, the changes at the *y* locus result in a novel pigmentation spot. **b**, Wing pigmentation patterns similar to *D. biarmipes* (left) or *D. guttifera* (right) evolved independently in other fly families (here Otitidae and Lauxaniidae).

Methods

Fly stock and maintenance

Flies were bred at 25 °C on Wheeler–Clayton[37] or cornmeal[38] medium. Constructs were transformed into *D. melanogaster yw* mutants as described previously[39,40]. The CantonS strain was used as wild-type *D. melanogaster*. *Drosophila pseudoobscura*, *Drosophila biarmipes* and *Drosophila guttifera* stocks were obtained from the Tucson stock centre (stock numbers 14011-0121.94, 14023-0361.01 and 15130-1971.10, respectively). All mature *D. biarmipes* males of this stock exhibited the wing spot. The *en-Gal4* and *UAS–GFP* stocks were obtained from the Bloomington *Drosophila* stock centre.

Immunochemistry

Pupal wings (70 h after puparium formation), still attached to the fly, were allowed to unfold in water after removal of the pupal cuticle. Flies were transferred to phosphate-buffered saline (PBS), in which the wings were cut off with a razor blade. Wings were fixed flat for 15 min between a slide and a coverslip in 4% formaldehyde PBT (PBS containing 0.03% Triton X-100), transferred on ice to a scintillation vial in the fixing solution for a further 15 min, sonicated briefly in the fixative with a Branson 200 ultrasonic cleaner, fixed for a further 30 min, washed with PBT, blocked for 1 h in PBT containing 1% bovine serum albumin, stained with a rat anti-*yellow* or a rabbit anti-*ebony* primary antibody[19] and revealed respectively with a fluorescein isothiocyanate (FITC)-conjugated anti-rat antibody or FITC-conjugated anti-rabbit IgG antibody (Jackson Immunoresearch).

Cloning

The *D. biarmipes y* locus sequence was amplified by direct and inverse polymerase chain reaction (PCR; details are available from the authors on request). The entire 5′ region was amplified by PCR with primers designed in the coding sequences of *y* and the closest gene upstream of *y* in *D. melanogaster* (*CG3777*; ref. 41). All *y* fragments for reporter constructs were cloned into a customized version of the P-based transformation vector[42] from which one of the two gypsy insulators had been removed and a new polylinker had been added. Fragments from *D. melanogaster* and *D. pseudoobscura* were amplified by PCR from genomic DNA and specific primers designed using available genome sequences[43,44] (see Supplementary Table 1 for primer sequences).

Biochemistry

The *D. melanogaster* Engrailed homeodomain sequence was cloned into the glutathione *S*-transferase (GST) gene fusion vector pGEX-3X (Amersham Bioscience). The GST fusion protein was purified by affinity chromatography[45]. DNA probes for electrophoretic mobility-shift assays were double-stranded oligonucleotides labelled with ^{32}P by end-filling in at both ends with the Klenow fragment of DNA polymerase I. Single-stranded oligonucleotides were annealed at a final concentration of 0.1 μM in 10 mM Tris-HCl pH 7.5 containing 0.1 M NaCl and 1 mM EDTA. Sequences of the oligonucleotide pairs were as follows: native sequences, 5′-TTTCCGCCTAATTGATG-3′ and 5′-TTTCATCAATTAGGCGG-3′, 5′-TTTTGCCAATCATTTTT-3′ and 5′-TTTAAAAATGATTGGCA-3′; mutated versions, 5′-TTTCCGCCTcccTGATG-3′ and 5′-TTTCATCAgggAGGCGG-3′, TTTTGCCgggCATTTTT-3′ and 5′-TTTAAAAATGcccGGCA-3′. Labelled probes were purified with G50 Sephadex beads (Sigma) on chromatography columns (Bio-Rad). DNA-binding assays, competition experiments and gel migrations were performed with 10–15 fmol of labelled probes (about 10^4 c.p.m.) following a published protocol[26]; they were pre-run for 0.5 h and run for 1.5 h at 4 °C on 8% native polyacrylamide minigels in 0.5 × Tris/borate/EDTA buffer pH 8.3. Non-specific competitor consisted of herring sperm DNA (Sigma) and the specific competitor was as used elsewhere[26].

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Wing imaging

Adult wings were mounted flat in Hoyer's medium[38] and processed for bright-field imaging with a 4× or 10× dry lens on a Zeiss Axiophot microscope equipped with a Kontron charge-coupled device camera. For all reporter lines, pupal wings 70–90 h after puparium formation were mounted flat between a slide and a coverslip in PBT, without fixation, and imaged immediately with an Optiphot confocal microscope (Nikon) equipped with a 4× dry lens and a BioRad 1024 system. Antibody-stained preparations were mounted in glycerol and imaged.

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