SCIENTIFIC REPORTS

OPEN

Pigmentation pattern and developmental constraints: flight muscle attachment sites delimit the thoracic trident of *Drosophila melanogaster*

Received: 9 January 2018
Accepted: 20 March 2018
Published online: 28 March 2018

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In their seminal paper published in 1979, Gould and Lewontin argued that some traits arise as by-products of the development of other structures and not for direct utility in themselves. We show here that this applies to the trident, a pigmentation pattern observed on the thorax of *Drosophila melanogaster*. Using reporter constructs, we show that the expression domain of several genes encoding pigmentation enzymes follows the trident shape. This domain is complementary to the expression pattern of *stripe (sr)*, which encodes an essential transcription factor specifying flight muscle attachment sites. We demonstrate that *sr* limits the expression of these pigmentation enzyme genes to the trident by repressing them in its own expression domain, *i.e.* at the flight muscle attachment sites. We give evidence that repression of not only *yellow* but also other pigmentation genes, notably *tan*, is involved in the trident shape. The flight muscle attachment sites and *sr* expression patterns are remarkably conserved in dipterans reflecting the essential role of *sr*. Our data suggest that the trident is a by-product of flight muscle attachment site patterning that arose when *sr* was co-opted for the regulation of pigmentation enzyme coding genes.

Body pigmentation is at the interface between the organisms and their environment and fulfils many ecologically relevant functions. Indeed, the adaptive role of pigmentation seems often so obvious – *i.e.* in crypsis, mimicry, aposematism, mate recognition, UV protection or thermoregulation - that it is generally assumed that the observed patterns have been selected. Direct selection on pigmentation has been demonstrated in many cases, such as the famous industrial melanism of the peppered moth, *Biston betularia*, whose molecular basis has recently been identified[1,2]. Pigmentation patterns can also be the object of a trade-off between opposite selection forces. For example, in guppy *Poecilia reticulata* males, pigmentation patterns result from a balance between selection for crypsis, an anti-predator strategy, and selection for conspicuousness to attract females[3]. Further evidence for selection is the convergent loss of pigmentation when selection is relaxed in organisms living in the absence of light, such as cave animals[4,5]. However, since Gould and Lewontin's seminal paper in 1979[6], it is widely acknowledged that adaptationist explanations should sometime be used with caution and developmental constraints also taken into account. Gould and Lewontin used the example of the spandrels decorated with splendid mosaics between the arches supporting the dome of the basilica of Saint-Mark in Venice. Although the mosaics fit remarkably well on the spandrels, the spandrels were not designed for them but result from architectural constraints imposed by the structure supporting the dome. Thus, Gould and Lewontin argued that some biological traits arose as by-products of developmental constraints on a crucial trait and were not selected for their direct utility[6,7]. Hence, developmental constraints favour particular patterns or morphologies whereas they forbid others. A few studies on pigmentation have addressed this question by exploring morphospaces with artificial selection experiments[8,9]. The size of the eyespots on butterfly *Bicyclus anynana* wings responds to selection but

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their colour is more constrained as only coordinated changes of pigments are possible[8]. Consequently, only some area of the morphospace can be occupied. Furthermore, the eyespot pattern through the genus *Bicyclus* follows a similar path of diversification, suggesting that evolution is partly constrained and that selection (or drift) can operate only in particular directions[8].

Drosophila pigmentation is an interesting model to study the impact of developmental constraints[10]. This highly evolvable trait has been the focus of many studies analysing the genetic bases of morphological variation within and between species[11,12]. Body or wing pigmentation relies on the coordinated action of *trans*-regulatory factors on genes encoding pigmentation enzymes[13–20]. Most of these factors, for example Engrailed or Abdominal-B, are spatially restricted components of a deeply conserved regulatory landscape involved in development of many essential traits. Hence, pigmentation patterns can be interpreted as targets of indirect selection due to their association with another trait[21].

The trident is a melanic pattern observed on the dorsal thorax of *Drosophila melanogaster*[22]. In natural populations, the intensity of the trident shows clinal genetic variation, with darker tridents observed at higher latitudes in Europe, India and Australia and higher altitudes in India and Africa[23–26]. The variable intensity of the trident is therefore thought to be an adaptation to temperature and/or UV[24,25]. Differences in the intensity of the trident were shown to be linked to genetic variation in the pigmentation gene *ebony (e)*, which is less expressed in flies with a darker trident[26,27]. Besides, the trident is more clearly visible in an *e* mutant background[28]. Furthermore, the intensity of the trident is sensitive to developmental temperature[24]. This trait is therefore an example of phenotypic plasticity, "*the ability of a given genotype to produce different phenotypes in response to distinct environmental conditions*"[29]. Interestingly, in *Drosophila busckii*, a pattern similar to the trident is very clearly delineated on the thorax, being dark brown on a yellow background[22,30]. Thus, the intensity of the trident is variable, plastic and highly evolvable. By contrast, the developmental bases of its shape have attracted little attention. Regulatory genes, whose mutation modifies it, are the most promising candidates to address the developmental constraints exerted. Mutation in the *stripe* gene (sr^1 allele) affects the shape of the trident[31]. This gene encodes an Egr-like zinc-finger transcription factor specifying epidermal tendon cells, to which muscles attach[32–35]. Therefore, *sr* expression labels the flight muscle attachment sites on the pupal thorax[32,35]. The expression pattern of *sr* on the pupal thorax[36] seems complementary to the trident, which suggests that *sr* might delimit the trident by repressing melanin production on the thorax. Using reporter constructs made with the regulatory sequences of pigmentation enzyme coding genes, we investigate here the role of *sr* in the establishment of the trident's pattern and its possible relationship with the positioning of the flight muscles. Using mutants, we show that *sr* represses the expression of several pigmentation enzyme-coding genes in the thorax epidermis, thus shaping the trident. The flight muscle attachment sites and *sr* expression pattern are remarkably conserved in dipterans, reflecting the essential role of *sr*[36,37]. Our data suggest that the shape of the trident is a by-product of flight muscle attachment site patterning that arose when *sr* was co-opted for the regulation of pigmentation enzyme-coding genes.

Results and Discussion

stripe represses melanin production on the thorax. The pattern of the trident varies among *Drosophila melanogaster* lines, being absent in some of them (w^{1118}) whereas clearly visible in others (*41Jd)* (Fig. 1a,b). In *ebony* mutant (e^1), the trident is very visible (Fig. 1c) as previously reported[28]. The trident is not limited to *Drosophila melanogaster* as it is reminiscent of the pigmentation pattern observed in another *Drosophila* species, *Drosophila busckii* (Fig. 1d).

Apparent complementarity between *sr* expression pattern and the trident in the pupal thorax[36] suggests that *sr* might be involved in trident patterning. To address this question, we took advantage of the sr^1 allele of *sr*. This is a very old allele, as it is reported to have been isolated by Calvin Bridges (1889–1938)[31]. In sr^1 flies, the trident is replaced by a broad longitudinal stripe, hence the name of the gene[31]. It is only thanks to the much more recent description of the *sr* expression pattern and to the characterization of the sr^1 allele that this phenotype can be interpreted. It was shown that sr^1 is a regulatory mutant, which loses the most dorsal domains of *sr* expression on the thorax[36]. To render the dark longitudinal stripe more visible, we combined sr^1 with the e^s hypomorphic allele[31]. The dark dorsal longitudinal stripe induced by sr^1 was perfectly visible in the e^s background (compare Fig. 1e,f). These data suggest that the dark longitudinal stripe is a modified trident, in which spaces between teeth are filled with melanin.

These spaces correspond to the dorsal domains of *sr* expression that are missing in sr^1, which suggests that *sr* shapes the trident by repressing melanin production. This repression could occur through different mechanisms implicating the expression, the stability or/and the activity of one or several pigmentation enzymes. Production of cuticle pigments involves many enzyme-coding genes arranged into a pathway[38,39]. Interestingly, among them, *ple* (encoding the Tyrosine Hydroxylase), *Ddc* (encoding the Dopa decarboxylase), *yellow (y)*, and *e* were shown previously to be expressed in the trident[28,40,41]. Over-expression of *y* in the dorso-medial domain in an *e* mutant background is sufficient to generate a homogenous black pigmentation[28]. Thus, restriction of *y* expression to the trident is sufficient to explain its delimitation. However, in a y^1, e^s double mutant, the trident is still visible (Fig. 1g) as previously reported[28] and in a y^1; sr^1, e^s triple mutant, the longitudinal pattern typical of sr^1 is clearly visible (Fig. 1h). This implies that other pigmentation enzymes than *y* and *e* are involved in the patterning of the trident downstream of *sr*.

Expression of _stripe_ and _tan_ in the thorax are complementary. Most pigmentation enzyme genes were previously shown to be expressed in the trident[28,40,41]. However, expression of *tan* (*t)*, which encodes an enzyme involved in melanin synthesis, was never analysed in the thorax. Using a transgene, in which the expression of nuclear enhanced green fluorescent protein (nEGFP) was driven by the *t* abdominal enhancer *t_MSE*[42,43], we observed nEGFP expression in the trident (Fig. 2a), showing than *t_MSE* was also activated in this motif. To reveal *sr* expression, we used the enhancer trap line sr^{md710} *(sr-Gal4)*[36] and the *UAS-mCherry-NLS* transgene.

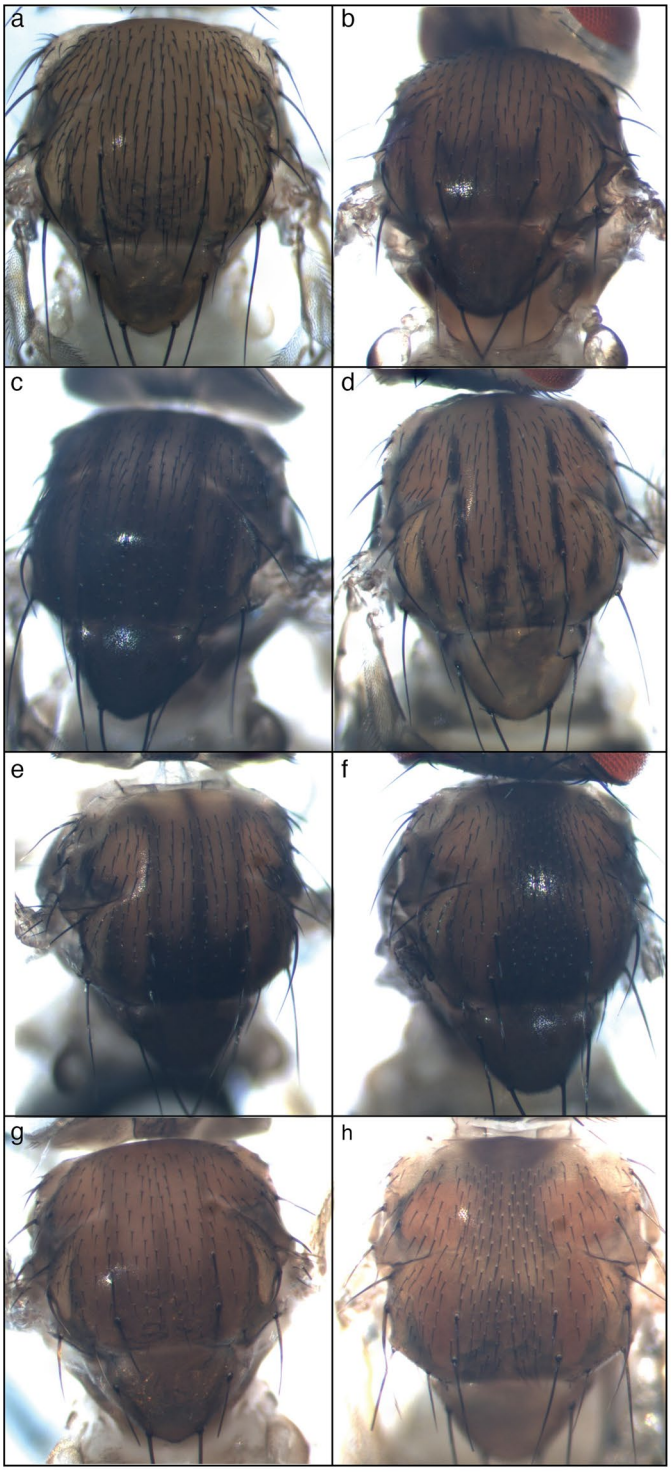

Figure 1. The trident pattern in *Drosophila*. (**a**) Absence of trident in w^{1118}. (**b**) Trident clearly visible in *41Jd*. (**c**) Dark trident of an *ebony* e^{1} mutant. (**d**) Distinctive trident in *Drosophila busckii*. (**e**) Trident of an *ebony* e^{s} mutant. (**f**) Dark longitunal stripe in an e^{s} sr^{1} double mutant. (**g**) Trident still visible in an y^{1}, e^{s} mutant background. (**g**) Longitudinal dark band in a y^{1}; e^{s} sr^{1} mutant.

mCherry expression was visible in the notum of pupae, where it precisely labelled the flight muscle attachment sites[36] (Fig. 2b). By combining (sr^{md710}, *UAS-mCherry-NLS*) with the *t_MSE-nEGFP* transgene, we observed a remarkable complementarity between the patterns of mCherry and nEGFP (Fig. 2c). All flight muscle attachment sites expressing mCherry corresponded to regions where nEGFP was absent, and a high nEGFP level was observed outside of the flight muscle attachment sites.

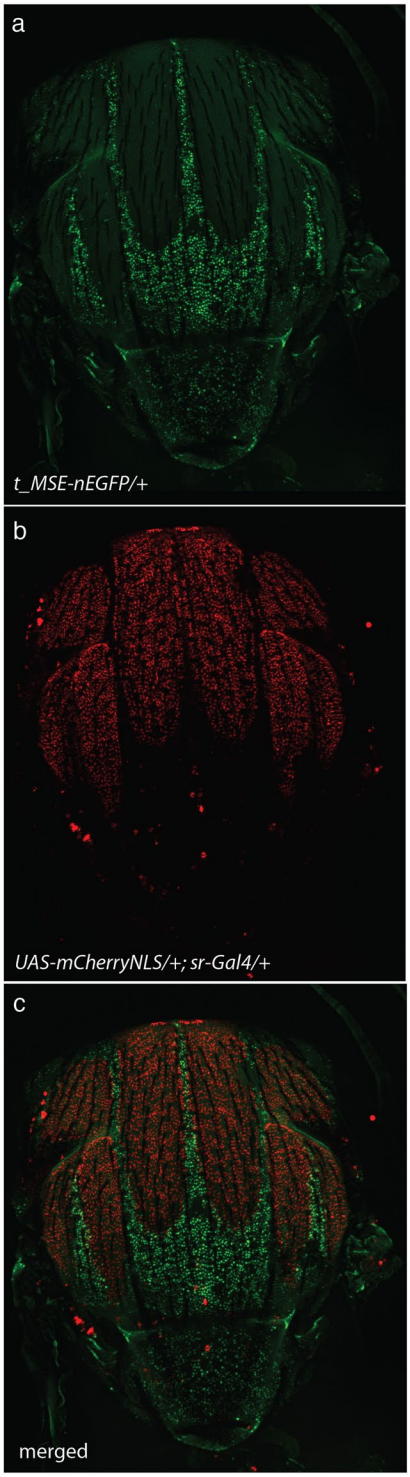

Figure 2. Complementary expression of *stripe* and *tan* in the thorax. Thorax of a *UAS-mCherry-NLS/t_MSE-nEGFP; sr-Gal4/+* freshly eclosed fly. (**a**) nEGFP showing the activity of *t* regulatory sequences; (**b**) mCherry showing *sr* expression; (**c**) Merge.

***stripe* delimits the shape of the trident by repressing multiple pigmentation genes.** Complementarity between *sr* and *t* expression domains on the thorax suggests that *sr* might repress the expression of *t*. This could also be the case for all pigmentation enzyme genes. Then, to draw up a precise and complete analysis of the expression domains of pigmentation enzymes in the thorax, as compared to the expression domain of *sr*, we used *Ddc-Gal4*[44] and *ple-Gal4*[45] associated with the *UAS-mCherry-NLS* transgene as well as transgenes expressing *nEGFP* under the control of *t*, *e* or *y* regulatory sequences[13,41,42]. In the control background, all reporters were expressed in the thorax with patterns that resembled the trident (Fig. 3a,c,e,g,i) suggesting that the expression

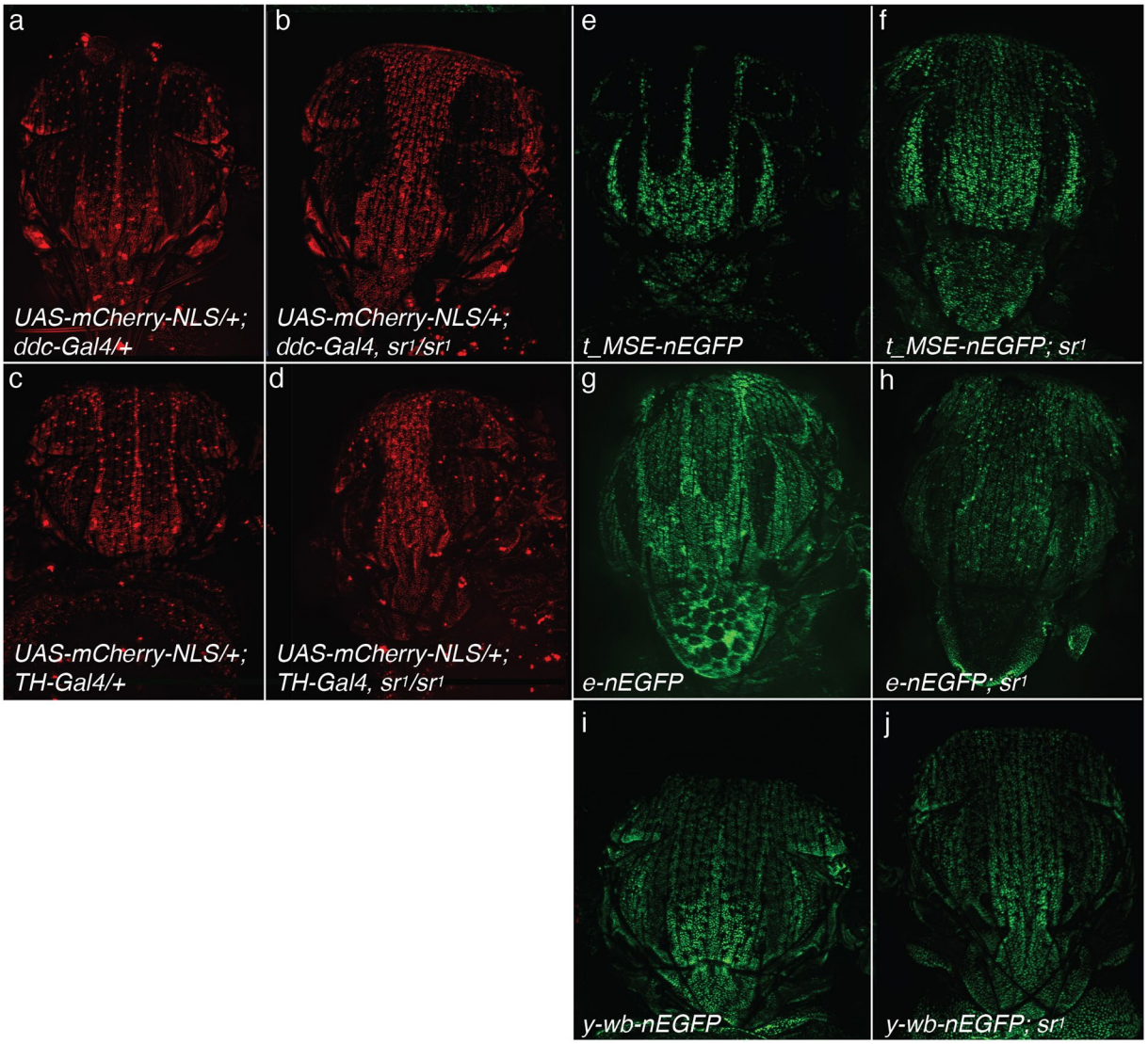

Figure 3. Effect of the sr^1 mutation on the expression of pigmentation enzyme coding genes. (**a**) *Ddc* expression in thorax of pharates visualized using *Ddc-Gal4* and *UAS-mCherry-NLS* transgenes (*UAS-mCherry-NLS/+; Ddc-Gal4*). (**b**) *Ddc* expression in a sr^1 background (*UAS-mCherry-NLS/+; Ddc-Gal4*, sr^1/sr^1). (**c**) *ple* expression visualized using *ple-Gal4* and *UAS-mCherry-NLS* transgenes (*UAS-mCherry-NLS/+; ple-Gal4/+*). (**d**) *ple* expression in a sr^1 background (*UAS-mCherry-NLS/+; ple-Gal4*, sr^1/sr^1). (**e**) *t* expression followed using the *t_MSE-nEGFP* transgene. (**f**) *t* expression in a sr^1 background (*t-MSE-nEGFP;* sr^1). (**g**) *e* expression visualized using the *e-nEGFP* transgene. (**h**) *t* expression in a sr^1 background (*e-nEGFP*, sr^1/sr^1). (**i**) *y* expression followed using the *y-wb-nEGFP* transgene. (**j**) *y* expression in a sr^1 background (*y-wb-nEGFP;* sr^1).

of pigmentation enzyme genes was constrained to this motif. By contrast, in the sr^1 background, expression of the reporters was extended dorsally and the trident motif disappeared (Fig. 3b,d,f,h,j as compared to a, c, e, g, i, respectively). These data indicate that *sr* represses *Ddc*, *ple*, *t*, *e* and *y* in the thorax. Hence, the shape of the trident likely reflects the spatial regulation of pigmentation enzyme coding genes by Stripe.

Conclusion

We show here that *sr* regulates several pigmentation enzyme-coding genes on the thorax, although we do not know whether this regulation is direct or not. The expression of *sr* is conserved in *Calliphora vicina*, a species that diverged from *Drosophila* lineage about 100 million years ago, suggesting that this gene is a member of a deeply conserved regulatory landscape[37]. *sr* plays an essential role in the establishment of flight muscle attachment sites, and conservation of its expression is mirrored by a remarkable conservation of the flight muscle apparatus in dipterans[46]. In contrast, a clear thoracic trident complementary to the flight muscle attachment sites is observed in only a few species of flies, notably in *Drosophila melanogaster*[22]. Our results suggest that the co-option of *sr* for the regulation of pigmentation enzyme coding genes has led to the generation of the trident, a pigmentation pattern complementary to the flight muscle attachment sites. Therefore, the shape of the trident primarily results

from a developmental constraint imposed by the flight muscle pattern. It is typically a "spandrel" in the sense of Gould and Lewontin (1979), a by-product of the development of another structure. The same applies to the position of large bristles (macrochaetes) on the thorax. Indeed, macrochaetes are excluded from the flight muscle attachment sites, and it was shown that the development of macrochaetes and tendon cells on the thorax are mutually exclusive[36].

The fact that the trident was originally an indirect target of selection does not exclude that it has later become a direct target of selection. Indeed, clinal variation in the intensity of the trident strongly suggests an adaptive role in thermoregulation[23,24,26]. However, natural selection has targeted variation in the intensity of the trident, rather than in its shape that is highly constrained by fixed muscle attachment sites. Furthermore, it is possible that the expression of pigmentation enzymes in the trident confers new properties to the thorax cuticle that are important for flight, such as flexibility or mechanical endurance.

Methods

Fly stocks. w^{1118} is an inbred line used as control. The line *41Jb* was established by Jean-Michel Gibert from a female caught in Marsais (France). The *Drosophila busckii* line was established from a female caught in Niort (France) and kindly provided by Dr Laure Teysset. The following stocks were obtained from the Bloomington Drosophila stock centre: e^1 (BL-1658), e^s (BL-498), *Ddc-Gal4* (BL-7009), *ple-Gal4* aka *TH-Gal4* (BL-8848), *sr-Gal4* aka sr^{md710} (BL-26663) and *UAS-mCherry-NLS* (BL-38425). The lines *y-wing-body-nEGFP*, *e-nEGFP (*containing the regulatory regions *ABC + intron)* and *t_MSE-nEGFP* were kindly provided by Dr. Sean Carroll's laboratory. Flies were grown on standard medium at 25 °C.

Image acquisitions. Thoracic cuticles of flies immerged in 75% ethanol were imaged with a binocular equipped with Leica DC480 digital camera, using the Leica IM50 Image Manager software. Stacks of 4–10 images were generated for each thorax. They were merged using Photoshop. Identical settings were used for all acquisitions.

Fluorescent images were acquired with a Macro-Apotome (Zeiss) with a 63 × objective on freshly decapitated flies immerged in PBS on an agarose substrate. Stacks were composed of around 75–115 pictures. Maximum intensity projections were created. Brightness and contrast were slightly adjusted in Photoshop.

Data availability. Data and materials used in this work are available on request.

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Acknowledgements

Flybase provided information useful for this study. We thank the Bloomington Drosophila Stock Centre and the Sean Carroll laboratory for fly stocks and Dr Laure Teysset for the *Drosophila busckii* line. We thank other members of the lab for fruitful discussions.

Author Contributions

J.M.G. designed the study, performed the experiments and analysed the data. J.M.G., E.M.V. and F.P. wrote the paper.

Additional Information

Competing Interests: The authors declare no competing interests.

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