Changes in global repression underlie the evolution of *Drosophila* abdominal pigmentation

Iván D. Méndez-González[1], **Thomas M. Williams**[2], **Mark Rebeiz**[1*]

***For correspondence:**
rebeiz@pitt.com (MR)

[1]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [2]Department of Biology, University of Dayton, Dayton, Ohio, USA

Abstract

Changes in gene regulation represent an important path to generate developmental differences affecting anatomical traits. Interspecific divergence in gene expression often results from changes in transcription-stimulating enhancer elements. While gene repression is crucial for precise spatiotemporal expression patterns, the relative contribution of repressive transcriptional silencer to regulatory evolution remains to be addressed. Here, we show that the *Drosophila* pigmentation gene *ebony* has mainly evolved through changes in the spatial domains of silencers patterning its abdominal expression. By precisely editing the endogenous *ebony* locus of *D. melanogaster*, we demonstrate the requirement of two redundant abdominal enhancers and three silencers that repress the redundant enhancers in a patterned manner. We observe a role for changes in these silencers in every case of *ebony* evolution observed to date. Our findings suggest that negative regulation by silencers likely has an under-appreciated role in gene regulatory evolution.

Introduction

Morphological evolution largely depends on changes in the expression of key developmental genes and their downstream target genes (***Carroll, 2008***; ***Prud'homme et al., 2006***). At the core of this process are *cis*-regulatory sequences known as enhancers, which are responsible for activating transcription in a specific spatiotemporal pattern (***Howard and Davidson, 2004***). Enhancers have been the focus of gene regulatory studies for several good reasons: they are typically discovered through reporter assays that test sufficiency and are most commonly found when a regulatory region is dissected. Although enhancers provide a good approximation of gene expression patterns, oftentimes they do not fully recapitulate the endogenous gene expression (***Barolo, 2012***). This highlights the importance of other types of regulatory sequences, including boundary elements (***Yokoshi et al., 2020***), Polycomb response elements (***Sengupta et al., 2004***), silencers (***Segert et al., 2021***), and sequences that lie at the outskirts of minimally defined enhancers (***Lopez-Rivera et al., 2020***), which interact with enhancers to accomplish precise spatiotemporal patterns of expression. Hence, a key task to understand the evolution of gene regulation is to pinpoint the influence of regulatory elements beyond enhancers, and every example provides key precedents that expand our conception of possible mechanisms.

Transcriptional repression has long been appreciated as an integral component of gene regulation (***Jacob and Monod, 1961***; ***Johnson, 1995***; ***Payankaulam et al., 2010***). Transcriptional silencers are *cis*-regulatory sequences that repress transcription from otherwise active promoters (***Halfon,***

2020). Recent evidence hints at the widespread prevalence of silencers in animal genomes (*Gisselbrecht et al., 2020*; *Pang and Snyder, 2020*; *Ngan et al., 2020*). However, the difficulty of genomically identifying and functionally characterizing these regulatory elements (*Halfon, 2020*) has limited our ability to test whether the modification of silencer function could be a general mechanism of morphological evolution (but see (*Johnson et al., 2015*)). Many mechanisms have been proposed for silencer function, from promoter-proximal mechanisms involving histone methylation, to distal elements capable of repressing at long ranges (*Segert et al., 2021*). Because of the long-range character of these elements, they are very difficult to identify by traditional reporter tests of sufficiency. Moreover, since these regulatory elements are able to completely shut down transcription in a patterned manner, they may represent a substantial source of phenotypically relevant genetic variation.

Drosophila melanic pigmentation represents a rapidly evolving trait that has provided many insights into regulatory and morphological evolution (*Rebeiz and Williams, 2017*). In particular, the *ebony* gene presents an intriguing model for understanding regulatory evolution because of its negative regulatory elements. *ebony* encodes an enzyme that decreases the production of black melanin pigments (*Wittkopp et al., 2002a*). In *D. melanogaster* males, *ebony* expression anticorrelates with the melanic pigments that adorn the adult abdomen, as it is restricted from the posterior part of the abdominal segments A2-A4 and down-regulated in entire A5 and A6 segments (*Rebeiz et al., 2009*). This expression pattern is controlled by multiple regulatory elements (*Figure 1*A) (*Rebeiz et al., 2009*; *Akiyama et al., 2022*). An upstream enhancer drives expression in the entire abdomen (hereafter referred as *eAct*) (*Rebeiz et al., 2009*). A promoter-proximal silencer represses ebony in the A5 and A6 segments of males (hereafter referred as *eMS*) (*Rebeiz et al., 2009*). And an intronic silencer represses ebony in the most posterior region of each segment (hereafter referred as *eSS*) (*Rebeiz et al., 2009*). Recently, it was found that *eAct* also functions as a dorsal midline silencer and that it controls ebony abdominal expression together with yet unidentified redundant enhancers (*Akiyama et al., 2022*).

ebony has been implicated repeatedly in the evolution of *Drosophila* pigmentation, and in all cases, *cis*-regulatory rather than coding changes were involved (*Rebeiz et al., 2009*; *Ordway et al., 2014*; *Johnson et al., 2015*; *Signor et al., 2016*; *Liu et al., 2019*). For instance, it was shown that the function of *eMS* is conserved in *D. prostipennis* and *D. yakuba* (*Ordway et al., 2014*; *Liu et al., 2019*), but not in *D. serrata* nor *D. santomea*, two species that secondarily lost male A5 and A6 melanic pigmentation (*Johnson et al., 2015*; *Liu et al., 2019*). Relatedly, this silencer's function was found to be polymorphic in *D. auraria* (*Johnson et al., 2015*). These findings are illustrative examples that morphological evolution can evolve via silencer inactivation to increase gene expression. The diversity of melanic pigmentation patterns (*Figure 1*B) that correlate with *ebony* abdominal expression (*Hughes et al., 2020*; *Signor et al., 2016*) presents an opportune system in which to investigate how regulatory evolution might recurrently proceed in the context of a complex regulatory architecture.

Here, we investigated the *cis*-regulatory evolution of *ebony* in *D. melanogaster* and relatives displaying a range of pigmentation phenotypes (*Figure 1*B). We found that changes in the function of silencers, rather than enhancers, have contributed to the most salient differences in *ebony* expression among *Drosophila* species with divergent melanic pigmentation. We identified a novel silencer that seemingly evolved within an abdominal enhancer, functionally equivalent silencers with different genomic locations, and spatial expansions in the domain of a silencer's function. Altogether, these data illustrate multiple manners in which differential negative regulation resulting from changes in the function of transcriptional silencers can contribute to phenotypic diversity.

Results

Redundant enhancers contribute to *ebony* abdominal expression in *D. melanogaster*

A recent study found that deleting the main abdominal enhancer (*eAct*) does not notably affect *ebony* expression, suggesting the presence of redundant enhancers (*Akiyama et al., 2022*). How-

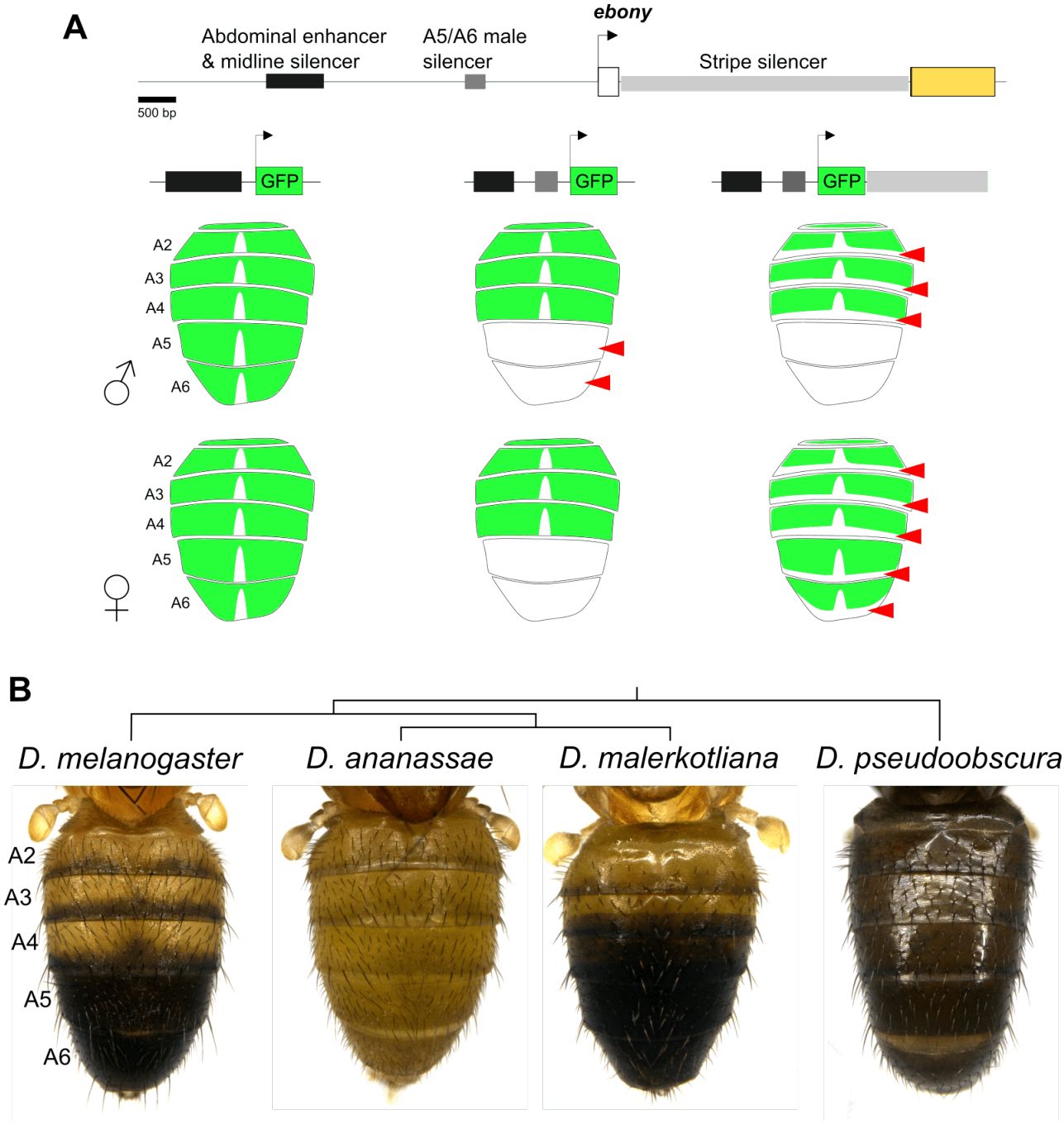

Figure 1. *ebony* abdominal expression is patterned by multiple regulatory elements. **(A)** Gene map of the *ebony* locus showing the location of known enhancers and transcriptional silencers active in the abdomen of *D. melanogaster*. The cartoons below represent the GFP reporter expression of the upstream enhancer alone and in combination with the two silencers. **(B)** Phylogeny showing the abdominal pigmentation of males from different *Drosophila* species.

ever, the number and location of such enhancers has not been determined. We used CRISPR-*Cas9* to create a series of deletions aiming to identify the redundant enhancer(s) (***Figure 2***B-C'). *ebony* null mutants develop a darker pigmentation compared to wild type controls (WT, ***Figure 2***A), setting the expectation that flies will become *ebony*-like once all redundant enhancers are removed. Deletion of *eAct* did not affect the abdominal pigmentation intensity (***Figure 2***D-D', J-K), confirming previous results (***Akiyama et al., 2022***). We wondered whether important sequences that maintain WT levels of *ebony* expression reside outside of the deleted region. To test this, we deleted an expanded region centered on *eAct*Δ (*eActB*Δ), and the entire upstream region (*eUps*Δ). Both deletions resulted in slightly darker flies compared to WT, although still considerably lighter than *ebony* null mutants (***Figure 2***E-F', J-K).

Even though these deletions only had a mild effect in the adult pigmentation, we wondered if they had any effect on *ebony* expression. We analyzed *ebony* mRNA in the abdomen of flies at the eclosion stage using *in situ* hybridization. While all deletion backgrounds showed WT levels of expression, deletions overlapping the *eAct* region resulted in *ebony* de-repression along the dorsal midline (***Figure 2–Figure Supplement 1***). These expression patterns correlate with the adult pigmentation of these lines in which the dorsal midline melanic stripe is erased (***Figure 2–Figure Supplement 1***B-I') and confirm the function of this region as a silencer (***Akiyama et al., 2022***). These results suggest that redundant enhancer(s) located outside the *ebony* upstream region work together with the element in the *eActB* region to ensure WT levels of expression in the abdomen.

To identify the redundant enhancer(s), we focused on a candidate region located within the first *ebony* intron (*eIN.4*, ***Figure 2***A). This region was identified as a putative abdominal enhancer in *Drosophila* species from the *ananassae* subgroup (***Signor et al., 2016***). Importantly this candidate region does not overlap with the intronic stripe silencer *eSS* (see below). We reasoned that a possible redundant enhancer could be identified by deleting this region in the *eActB*Δ or *eUps*Δ backgrounds. The deletion of the candidate region alone (*eIN.4*Δ) did not affect the pigmentation (***Figure 2***G-G'). However, both double deletions, *eActB+IN.4*Δ and *eUps+IN.4*Δ, resulted in much darker pigmentation compared to the single deletions and approaching to the pigmentation of *ebony* mutants (***Figure 2***H-K). Thus, *eIN.4* functions as a partially redundant enhancer working together with *eActB* to drive robust ebony expression in the abdomen.

Although we focused on the abdominal pigmentation, we noticed that other tissues including the head, thorax, legs, halteres, and wings of *eUps+IN.4*Δ had a darker pigmentation compared to WT (***Figure 2–Figure Supplement 2***). Enhancers responsible for *ebony* expression in these tissues have been mapped to the upstream region (***Rebeiz et al., 2009***). However, the pigmentation of these tissues in *eActB*Δ and *Ups*Δ appears WT (***Figure 2–Figure Supplement 2***). Thus, *eIN.4* represents a redundant enhancer that is active in multiple adult tissues. Altogether, these experiments revealed a complex mechanism for *ebony* regulation in which upstream tissue-specific enhancers collaborate with an intronic epidermal redundant enhancer to ensure robust expression in the adult cuticle.

ebony abdominal silencers are active in specific spatial domains

Gene reporter analysis suggests that *ebony* repression in the male A5 and A6 segments is mediated by a silencer referred as *eMS* (***Rebeiz et al., 2009***). To confirm the function of *eMS* in its endogenous context, we created a deletion targeting this region (***Figure 3***A). While the A5-A6 pigmentation was not affected in *eMS*Δ (***Figure 3***B-C, F), we observed higher *ebony* mRNA expression compared to WT as measured by *in situ* hybridization (***Figure 3***D-E). These experiments confirm that *eMS* is necessary to repress ebony in the A4 and A5 male segments. The lack of phenotypic effects can be explained by the high expression of genes with an opposite function to *ebony*, like *tan* and *yellow* (***Wittkopp et al., 2002b***; ***Camino et al., 2015***).

ebony expression is also repressed in the area where the posterior melanic stripes develop by an intronic silencer referred as *eSS* (***Rebeiz et al., 2009***). We narrowed down the exact location of this silencer using nuclear-localized Green Fluorescent Protein (or GFP) reporter constructs

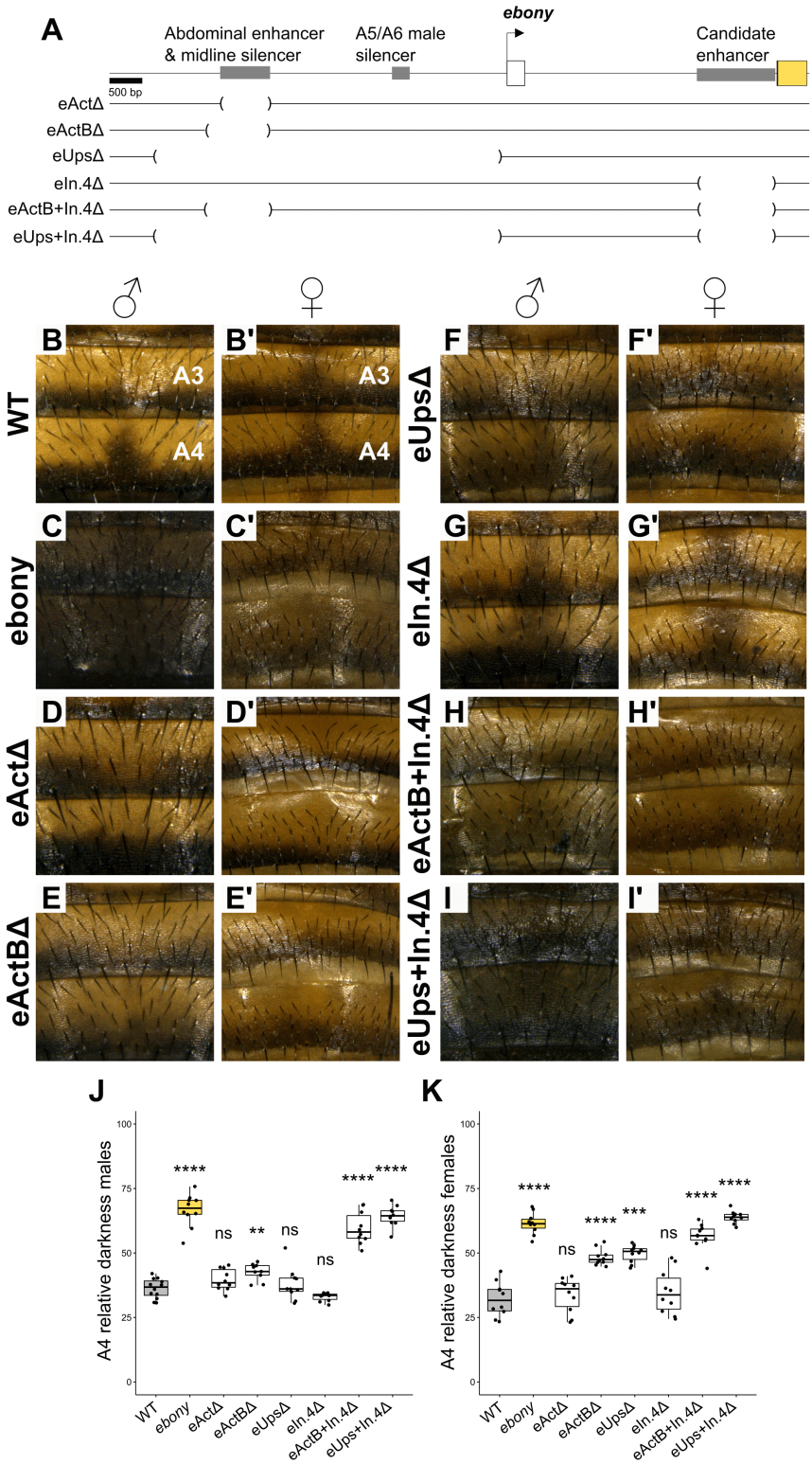

Figure 2. *ebony* abdominal expression is controlled by redundant enhancers. **(A)** Gene map of the *ebony* locus showing the location of the deletions created to identify redundant enhancers. **(B-I')** A3 and A4 pigmentation of WT, *ebony* null mutants, and deletion lines males and females. **(J-K)** Quantification of the A4 relative darkness of males **(J)** and females **(K)**. Significant differences are shown compared to WT. (Student's t test, ns = not significant, *p < 0.5, **p < 0.05, ***p < 0.005, ****p < 0.0005)

Figure 2–Figure supplement 1. *ebony* abdominal *mRNA* expression correlates with pigmentation phenotypes

Figure 2–Figure supplement 2. The redundant intronic enhancer is active in multiple adult tissues

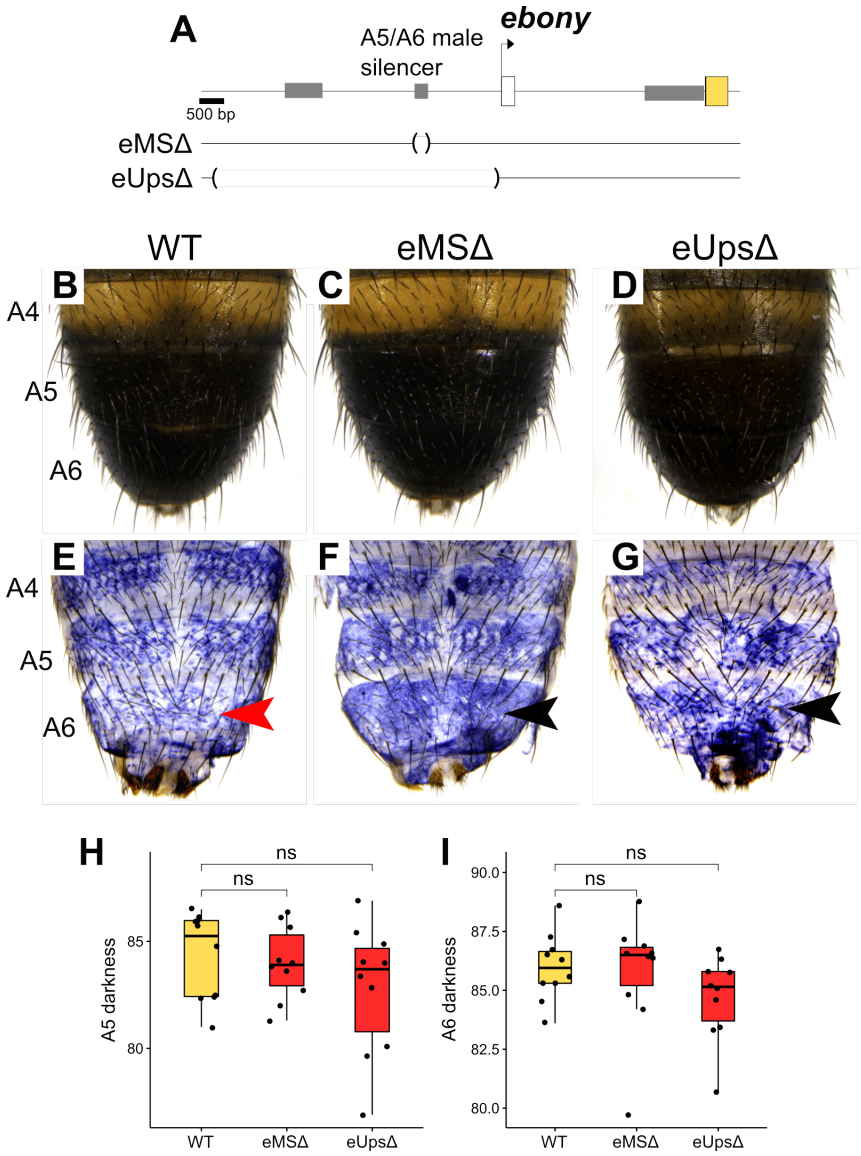

Figure 3. Necessity of the *ebony* A5/A6 male silencer. **(A)** Gene map of the *ebony* locus showing the location of the deletion targeting the A5/A6 male silencer (*eMS*Δ). **(B-D)** A4, A5, and A6 pigmentation of WT, *eMS*Δ, and *eUps*Δmales. **(E-G)** *in-situ* hybridization detecting *ebony mRNA* in A4, A5 and A6 segments of WT, *eMS*Δ, and *eUps*Δmales. Red and black arrowheads indicate low and increased levels of *ebony mRNA*, respectively. **(F)** Comparison of A5 and A6 darkness between WT, *eMS*Δ, and *eUps*Δmales. (Student's t test, ns = not significant).

Figure 3–Figure supplement 1. Identification of the stripe silencer within the first *ebony* intron

Figure 3–Figure supplement 2. Necessity of the *ebony* stripe silencer

containing fragments of the *ebony* first intron. A region of 1.5 kb located downstream of the *ebony* promoter (eUps+In.1) showed low GFP expression in the stripe area (***Figure 3–Figure Supplement 1***). The endogenous deletion of this region resulted in *ebony* de-repression in the stripe area and thinner melanic stripes compared to the WT (***Figure 3–Figure Supplement 2***), confirming that this region is *eSS*. Together, these experiments show that the silencers *eMS* and *eSS* are necessary and sufficient to repress the *ebony* redundant enhancers in specific spatial domains.

Changes in the function of silencers drive the evolution of *ebony* expression among *Drosophila* species

To understand how *ebony* expression has evolved, we analyzed its regulation in three additional *Drosophila* species. *ebony* has been identified as a major driver of pigmentation diversity within the *ananassae* species subgroup (***Signor et al., 2016***). Thus, we selected two species from this group with contrasting abdominal pigmentation, *D. ananassae* (non-melanic) and *D. malerkotliana* (A4, A5 and A6 melanic). We also included *D. pseudoobscura*, a completely melanic species which displays very low levels of *ebony* expression (***Hughes et al., 2020***) (***Figure 4***A). We created three reporter constructs for each species, containing the region orthologous to the upstream abdominal enhancer (*eAct*), the entire upstream region (*eUps*), and the upstream and first intronic region (*eUps+IN*, ***Figure 4***B). These constructs were tested for GFP activity in the A4-A6 segments of transgenic *D. melanogaster* males 24 hours (h) after eclosion.

We found that the activator region of *D. ananassae* drives reporter expression in all abdominal segments (***Figure 4***C). Qualitatively, this expression pattern did not change when the full upstream region (***Figure 4***D) or upstream together with the intronic regions were analyzed (***Figure 4***E). These results suggest that in *D. ananassae*, *ebony* abdominal expression is controlled by an upstream enhancer (***Figure 4***L).

For *D. malerkotliana*, we found that the activator and the upstream region drive uniform GFP expression in all abdominal segments (***Figure 4***F-G). This reporter activity does not recapitulate *ebony* the endogenous expression of *D. malerkotliana*, which is restricted from the A4, A5, and A6 segments (***Figure 4***B, (***Signor et al., 2016***)). However, when the intronic region was included, the expression in A5 and A6 was silenced (***Figure 4***H), suggesting the presence of an intronic A5-A6 male-specific silencer. The lack of A4 repression, which is observed in the *ebony* endogenous expression in this species, could result from changes in the trans landscape compared to *D. melanogaster*, or an unidentified A4 silencer. We noticed that the *D. malerkotliana eUps+IN* reporter also repressed GFP expression in the stripe area (***Figure 4***D, G). This suggests that this species contains intronic silencer(s) active in both the A5-A6 segments and in the stripe area. We hypothesized that the male silencer is located in an intronic region implicated in the pigmentation differences between *D. malerkotliana* and its sister species *D. malerkotliana pallens* (***Signor et al., 2016***), while the stripe silencer might be orthologous to the *D. melanogaster eSS*. GFP expression of a reporter containing the upstream and the candidate intronic regions (*eUps+IN.4*) was repressed in A5-A6, but not in the stripe area (***Figure 4–Figure Supplement 1***). Thus, the *IN.4* region contains the male silencer and might indeed underlie the pigmentation differences between *D. malerkotliana* and its sister species, while the stripe silencer seems to be conserved with respect to that of *D. melanogaster* (***Figure 4–Figure Supplement 1***). These results suggest that in *D. malerkotliana*, *ebony* abdominal expression is controlled by an upstream enhancer and at least two tissue-specific silencers (***Figure 4***M).

For *D. pseudoobscura*, we found that the activator region drives GFP expression in A4-A6 segments in a similar pattern to *D. ananassae* and *D. malerkotliana* (***Figure 4***I). This was surprising considering that the endogenous expression of *ebony* in *D. pseudoobscura* is almost undetectable (***Hughes et al., 2020***). However, when the full upstream region was analyzed, we found no GFP expression throughout the abdomen (***Figure 4***J). This suggests that *D. pseudoobscura* has a functional abdominal enhancer, which is repressed by a silencer located between this enhancer and the *ebony* promoter. When the upstream and intronic regions were analyzed together, we observed GFP expression only in A6 albeit at low levels (***Figure 4***K). We analyzed the reporter expression of

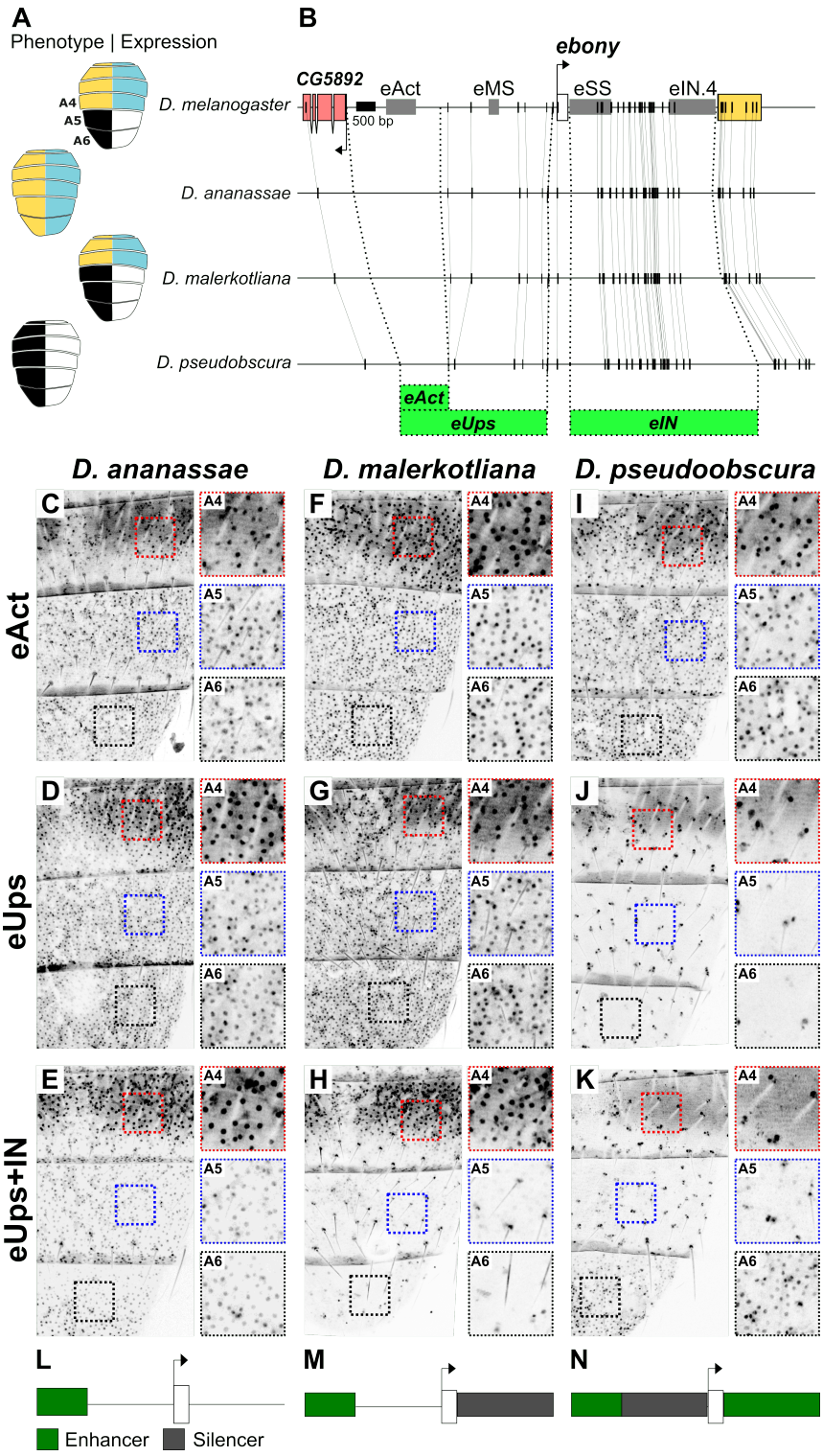

Figure 4. Changes in the location and function of transcriptional silencers among *Drosophila* species. **(A)** Cartoons representing the pigmentation phenotype (left) and the *ebony* expression pattern (right, blue color) of *D. melanogaster*, *D. ananassae*, *D. malerkotliana*, and *D. pseudoobscura*. **(B)** Gene map showing the location of the *ebony* CREs in *D. melanogaster* (top). Vertical lines indicate conserved regions, while dashed lines indicate the fragments used to create reporter constructs (green, bottom). **(C-K)** GFP expression patterns of the indicated transgenic reporters in the posterior abdominal segments A4-A6. Insets show magnified regions for A4 (red square) A5 (blue square), and A6 (black square). All flies were imaged 24 h post ecolosion. **(L-N)** Inferred *ebony* regulatory architecture showing the approximate location of abdominal enhancers (green) and silencers (gray).

Figure 4–Figure supplement 1. The *ebony* male and stripe silencers of *D. malerkotliana* are located in distinct intronic regions

Figure 4–Figure supplement 2. Enhancer activity of the *ebony* intronic region from *D. pseudoobscura*

Figure 4–Figure supplement 3. The melanic dorsal midline is novel to *D. melanogaster*

the intronic region alone and found it to be A6 specific (***Figure 4–Figure Supplement 2***). These data suggest that the low *ebony* abdominal expression of *D. pseudoobscura* (***Hughes et al., 2020***) results from a silencer that represses *eAct* in all abdominal segments but seems unable to repress the A6 intronic enhancer (***Figure 4***N).

Evolution of the melanic dorsal midline through the gain of a novel silencer

The melanic stripe that forms along the dorsal midline in *D. melanogaster* (***Figure 1***B) is regarded as characteristic of species within the subgenus *Sophophora* (***Markow and O'Grady, 2005***). However, we have not observed this pigmentation trait in species from the *ananassae* or *montium* subgroups. Given that the formation of the melanic dorsal midline requires *ebony* repression via the silencer activity of *eAct* (***Figure 2–Figure Supplement 1***) (***Akiyama et al., 2022***), we wondered about the evolution of this silencer function. We found that the *eAct* transgenic reporter of the three species studied here drive robust GFP expression along the dorsal midline (***Figure 4–Figure Supplement 3***A), suggesting that none of these species contain a functional midline silencer. To expand our phylogenetic sample, we analyzed the ebony midline expression and silencer function using published data for *D. prostipennis*, *D. serrata*, *D. auraria*, *D. yakuba*, and *D. santomea* (***Ordway et al., 2014***; ***Johnson et al., 2015***; ***Liu et al., 2019***). None of these species showed evidence of *ebony* midline repression or of a functional midline silencer (***Figure 4–Figure Supplement 3***B). Thus, the silencer function of *eAct* seems to be novel to *D. melanogaster* and may have contributed to the evolution of the melanic dorsal midline.

Discussion

The importance of silencers for patterning gene expression in metazoans has long been recognized (***Brand et al., 1985***). However, this mode of negative regulation has been difficult to study due to limited examples and heterogeneous mechanisms of action (***Halfon, 2020***; ***Segert et al., 2021***). We showed that multiple silencers are required for patterning spatial and sex-specific *ebony* abdominal expression, and that changes in the function of these silencers have resulted in altered expression patterns contributing to variation in abdominal pigmentation. Interestingly, the ability of *ebony* silencers to antagonize redundant enhancers appears to be case-specific. Below, we reconstruct the evolution of the *ebony* regulatory architecture and discuss how current experimental practices might obscure the significance of silencer evolution in the study of regulatory evolution (***Figure 5***).

Evolutionary history of a complex regulatory architecture

D. melanogaster has evolved a complex assemblage of two enhancers and three tissue-specific silencers required for shaping *ebony* abdominal expression. Comparative analysis of our reporter constructs suggests that each *ebony cis*-regulatory element has a unique evolutionary history (***Figure 5***A). The upstream enhancer (*eAct*) seems to have evolved, at least, in the common ancestor of the *melanogaster-obscura* species groups. However, the dual function of this region as a dorsal midline silencer (***Akiyama et al., 2022***) appears novel to *D. melanogaster*, where it seems to have contributed to the evolution of the melanic dorsal midline. Regarding *eMS*, we propose that the common ancestor of the *melanogaster-obscura* groups possessed a functional upstream silencer, as *D. pseudoobscura* also contains an upstream silencer (which is active in both sexes). After the divergence of these lineages, this silencer acquired a male-specific function specifically in the *melanogaster* group, which coincides with the evolution of male-specific melanic pigmentation (***Jeong et al., 2006***). However, the *ananassae* subgroup seems to have gained an intronic male-silencer, while losing the upstream silencer activity. Interestingly, the *D. malerkotliana* male-silencer maps to the same genomic region as the redundant intronic enhancer of *D. melanogaster*. Although challenging, future work involving these intronic regulatory elements might help to elucidate how enhancer logic and silencer logic could interconvert.

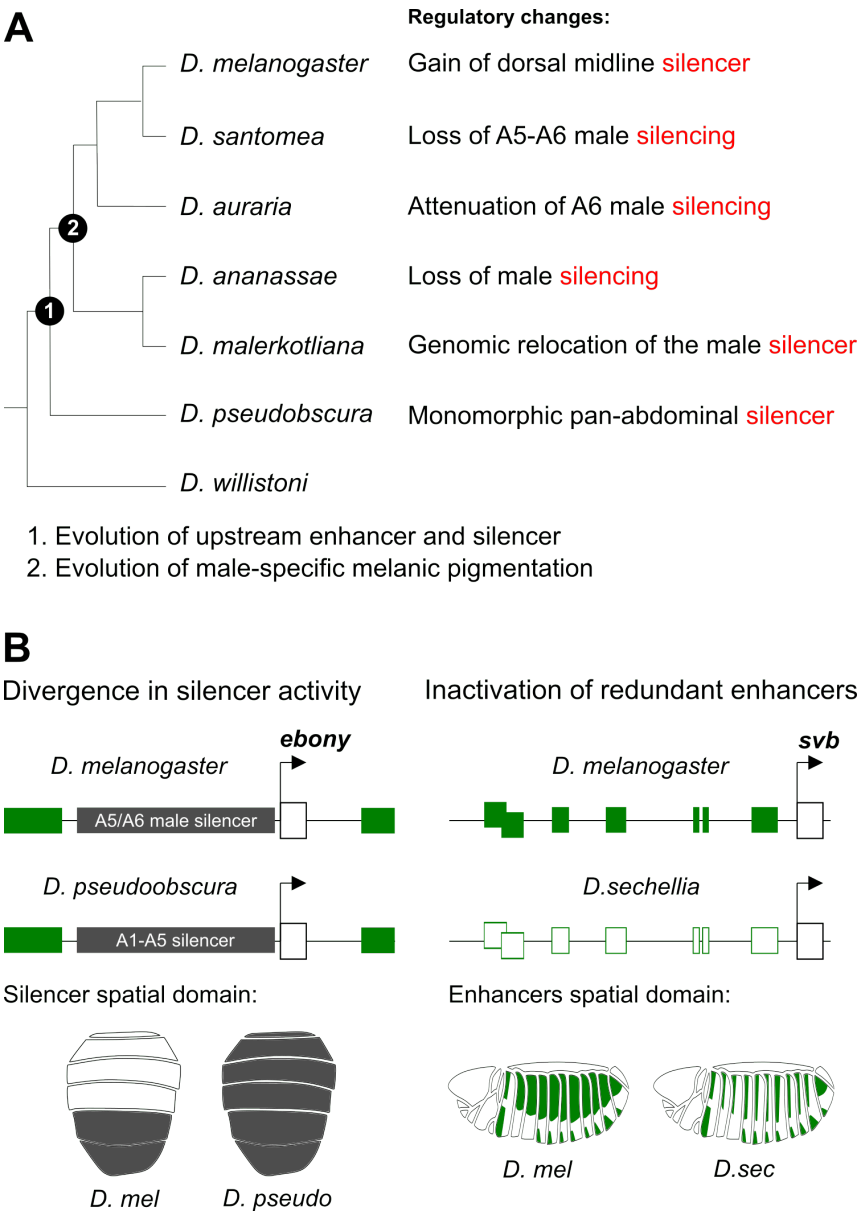

Figure 5. Changes in the function of silencers drive the regulatory evolution of *ebony* abdominal expression. **A)** Summary of *ebony* regulatory changes in *Drosophila* species from this and previous studies, and in relation to the evolution of male-specific melanic pigmentation. The second column shows the inferred regulatory changes in each species. **B)** Two possible mechanisms leading to morphological evolution through loss of tissue-specific expression. Left, ebony repression by an abdominal silencer contributes to the dark pigmentation of *D. pseudoobscura*. Right, inactivation of *shavenbaby* (*svb*) enhancers decreases the number of trichomes in *D. sechellia* compared to *D. melanogaster*. In both panels, green boxes represent functional enhancers.

Loss of expression by increased negative regulation of a functional enhancer

The characteristic dark pigmentation of *D. pseudoobscura* correlates with low *ebony* expression and high *yellow* expression (**Hughes et al., 2020**; **Wittkopp et al., 2002b**). Unexpectedly, we found that this species has a functional *ebony* abdominal enhancer that is is likely homologous to the *D. melanogaster eAct* . However, a silencer active throughout the abdomen strongly represses this enhancer. Of note, the ubiquitous silencer of *D. pseudoobscura* is not able to repress the A6 intronic enhancer. This provides an important exception to the observed trend that *ebony* silencers are global rather than selective. Silencers appear to comprise multiple functional classes, characterized by distinct associated proteins and interactions with other regulatory elements (**Segert et al., 2021**). **Gisselbrecht et al.** (**2020**) found that embryonic silencers bound by the Snail repressor likely function by preventing nearby enhancers from activating the transcription of target genes. Snail-unbound silencers, on the contrary, seem to loop directly to promoters where they recruit repressive activities. The second class, thus, would result in repression regardless of enhancer redundancy. Investigating the mechanisms of the *ebony* enhancers and silencers may resolve how differences in the mode of silencer action are encoded.

Morphological evolution often results from loss of tissue-specific expression following enhancer inactivation (**Chan et al., 2010**; **Jeong et al., 2006**; **Prud'homme et al., 2006**). An extreme example is the evolution of trichome patterns in *D. sechellia*, which involved the parallel inactivation of multiple enhancers of the *shavenbaby* gene (**McGregor et al., 2007**). Our results thus provide a distinct counterexample in which the dark pigmentation of *D. pseudoobscura* might have evolved through strong repression of *ebony* while preserving enhancer functionality (**Figure 5**B). These two paths to evolution would appear to differ in the number of required steps, as inactivation of multiple enhancers would likely involve more mutations than changes to a global silencer. However, it is important to remember that experimental biases towards enhancer studies, as discussed below, may skew our interpretations.

Transcriptional silencers and morphological evolution

Is the trend of silencer evolution at *ebony* an exception? It is our opinion that the *Drosophila* abdomen reflects an opportune system in which to notice repressive mechanisms that may be more prevalent than currently expected. Compared to microscopic tissues with three-dimensional complexity such as the embryo or imaginal disc, the abdomen is a relatively simple two-dimensional canvas in which even slight deviations of a reporter gene pattern from the endogenous expression pattern can be easily detected. Thus, a gene subject to silencer regulation, such as *ebony* would be easier to detect in this system.

The enhancer-centric way that gene regulatory evolution is studied is also skewed to overlook the potential role of silencers. When a difference in gene expression is found between distantly related species, the only way to determine whether those differences are caused by *cis*-regulatory evolution is to find the responsible enhancer(s) and ask whether they have differing activities using gene reporter constructs tested in a common genetic background (**Rebeiz and Williams, 2012**; **Rebeiz et al., 2015**). If the reporter genes recapitulate differences in expression observed within these species, such a result would be consistent with a *cis*-regulatory basis for these evolutionary differences. On the other hand, interspecific differences in enhancer-reporter expression are often attributed to *trans*-regulatory evolution. And yet, it may well be that these differences are actually encoded by *cis*-regulatory changes affecting silencer function. Considering the relative difficulty of finding and testing silencers (**Halfon, 2020**; **Segert et al., 2021**), it stands to reason that these modes of regulatory evolution are likely to be much more common than previously appreciated. Genomic surveys of open chromatin may offer an avenue to identify silencers and other regulatory elements. Indeed, in the butterfly wing, the endogenous deletion of an ATAC-seq peak region was associated with expanded expression, consistent with silencer function (**Lewis et al., 2019**). Thus, as the field of evolutionary-developmental biology seeks to further understand the *cis*-regulatory basis for morphological evolution, it will almost certainly have to contend with silencers and other

long-distance interacting elements as needles in a vast regulatory sequence's haystack.

Methods and Materials

Drosophila strains and culture conditions

Fly stocks were reared using standard culture conditions. Wild type species used in this study were obtained from the University of California, San Diego *Drosophila* Stock Center (now known as The National *Drosophila* Species Stock Center at Cornell University) (*Drosophila ananassae* #14024–0371.13, *Drosophila malerkotliana* #14024–0391.00, *Drosophila pseudoobscura* #14011–0121.87). The following were obtained from the Bloomington *Drosophila* stock center: *nos-Cas9 (attP40)* (#78781), *cre(III)* (#1501), double balancer (#3703), and ϕ *C31(X)* (#34772). A *D. melanogaster yellow white* (*yw*) strain that was isogenized for eight generations and was used to normalize the backgrounds of GFP reporter transgenes. The line used as WT was created by crossing the *yw* strain with the double balancer line and was used to compare with CRISPR-*Cas9* engineered lines.

CRISPR-Cas9 genome editing

Design of single guide RNAs (sgRNAS)

To avoid possible off-target effects, sgRNAs were designed using the CRISPR Optimal Target Finder (http://targetfinder.flycrispr.neuro.brown.edu/) and synthesized in vitro. Briefly, 20 nt target-specific primers were designed containing the T7 promoter sequence (upstream) and an overlap with the sgRNA scaffold (downstream). Each target-specific primer was combined with three primers for an overlap extension PCR (0.4 mM each) to generate a 130 bp DNA template. After purification, the template was used for in vitro transcription using EnGen sgRNA synthesis Kit (NEB), and the reaction was cleaned up using the MEGACLEAR Transcription Clean-Up KIT (Thermo). See key resources table for primers sequences.

Donor vectors for homologous directed repair

Homology arms (1.5-2 kb each) were amplified from the D. melanogaster strain to be injected and inserted into plasmids containing fluorescent eye markers using NEBuilder Hi-Fi DNA assembly (NEB). See key resources table for primers sequences and donor plasmids.

Drosophila microinjections

CRISPR-*Cas9* injections were performed in house following standard protocols (ref). All concentrations are given as final values in the injection mix. For the *ebony* loss of function strain, we injected a mix containing a sgRNA targeting the first exon (100 ng/µl), and the plasmids *pCRISPaint-sfGFP-3xP3-RFP* (Addgene 127566) and *pCFD5-frame_selector_0,1,2* (Addgene 131152; 400 ng/µl each) into nos-*Cas9* (attp40). This resulted in the insertion of *pCRISPaint-sfGFP-3xP3-RFP* in the first exon via non-homologous end joining, leading to a loss of function allele (***Bosch et al., 2020***).

For deletions of the *ebony* non-coding regions, we injected a mix containing the donor vector (500 ng/µl) and one to three sgRNAs flanking each side of the targeted region (100 ng/µl each). For eActΔ, eMaleSilΔ, and eActB + In.4Δ, and eUps + In.4Δ, the EnGen Spy Cas9 NLS (NEB) was added to the mix. eActBΔ, eUpsΔ, and eIn.4Δwere obtained by injecting into the nos-Cas9(attP40) strain (BDSC 78781). The progeny of each injected fertile individual was screened for dsRed, RFP or GFP fluorescence in the eyes and the correct genomic incorporation of this marker was confirmed by PCR followed by sequencing (see key resources table for primers sequences). Transformant individuals were crossed with a *yw* strain to remove the nos-*Cas9* transgene, and with a third chromosome balancer strain (BDSC 3703) to produce a stable homozygous line.

Pigmentation quantification

Representative images of the adult pigmentation patterns of each genotype were prepared from 7- to 8-day-old adults. To quantify the abdominal pigmentation, 10 cuticle preparations (REF) from adult flies were used for each genotype and sex. Briefly, flies were aged to 7-8 days old and stored

for 2-3 days in ethanol 75% before dissection. Abdominal cuticles were cut through the dorsal midline, which is therefore not visible in the preparations. After dissection, cuticles were mounted in PVA mounting medium (Bioquip). Cuticle preparations were imaged using a Leica M205C Stereo Microscope with a DFC425C camera. Image analysis was performed in ImageJ (***Abràmoff et al., 2004***). Images were blinded using the ImageJ extension LabCode, a region of interest was drawn in the anterior part of each abdominal segment using the freehand selection and the mean grayscale darkness was obtained. The relative darkness was calculated as: (255-grayscale darkness)/255 × 100 (***Rebeiz et al., 2009***). Boxplots were created using the R (***R Core Team, 2022***) packages ggplot2 (***Wickham, 2016***) and ggpubr (***Kassambara, 2020***).

in-situ hybridization

in-situ hybridization was performed as described in (***Liu et al., 2019***) with small modifications. In brief, flies were collected no more than 30 minutes after eclosion, dissected in cold PBS, and fixed in PBS containing 4% paraformaldehyde (E.M.S. Scientific) and 0.1% Triton X-100. PCR was performed to generate an RNA probe template that had a T7 promoter appended through primer design (see key resources table for primers sequences used). Digoxigenin-labeled probes were generated using a 10X Dig labeling mix (Roche Diagnostics) and T7 RNA polymerase (Promega). Dissected samples were probed using an in-situ hybridization robot (Intavis).

GFP transgenic reporters

ebony non-coding regions from different species were amplified via PCR and cloned into the S3AG vector using NEBuilder Hi-Fi DNA assembly (NEB) (Table X). *D. melanogaster* transformant lines were generated by ΦC31 mediated site specific recombination into the 51D insertion site on the second chromosome. Injections were performed by BestGene Inc. For all reporters, samples were aged 24h after eclosion and mounted in halocarbon oil 700 (SIGMA). Images were taken using an Olympus Fluoview 1000 confocal microscope. Samples were imaged with standard settings in which the brightest samples were not saturated. GFP expression was quantified using ImageJ (***Abràmoff et al., 2004***). The pixel intensity of a squared region was measured in the anterior part of A4, the posterior part of A4 and in the middle part of A5. The stripe silencing activity was calculated as the intensity of the posterior part of A4 divided by the intensity of the posterior part of A4. The A5 silencing activity was calculated as the intensity of the A45 segment divided by the intensity of the A4 segment.

Acknowledgments

The authors thank the members of the M.R. laboratory for their comments and discussion on the manuscript. We thank the Bloomington stock center, the National Drosophila Species Stock center for fly stocks, and Stephanie Day for help cloning reporter lines. This work was supported by funding from the NIH (R35GM14196 to MR) and the NSF (IOS2211833 to TMW)

Competing interests

No competing interests declared.

Author Contributions

Iván D. Méndez González, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing - original draft, Writing - review and editing; Thomas M. Williams, Conceptualization, Supervision, Funding acquisition, Methodology, Writing - review and editing; Mark Rebeiz, Conceptualization, Supervision, Funding acquisition, Methodology, Writing - review and editing.

References

Abràmoff MD, Magalhães PJ, Ram SJ. Image processing with ImageJ. Biophotonics international. 2004; 11(7):36–42.

Akiyama N, Sato S, Tanaka KM, Sakai T, Takahashi A. The role of the epidermis enhancer element in positive and negative transcriptional regulation of ebony in Drosophila melanogaster. G3. 2022; 12(3):jkac010.

Barolo S. Shadow enhancers: frequently asked questions about distributed cis-regulatory information and enhancer redundancy. Bioessays. 2012; 34(2):135–141.

Bosch JA, Colbeth R, Zirin J, Perrimon N. Gene knock-ins in Drosophila using homology-independent insertion of universal donor plasmids. Genetics. 2020; 214(1):75–89.

Brand AH, Breeden L, Abraham J, Sternglanz R, Nasmyth K. Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell. 1985; 41(1):41–48.

Camino EM, Butts JC, Ordway A, Vellky JE, Rebeiz M, Williams TM. The evolutionary origination and diversification of a dimorphic gene regulatory network through parallel innovations in cis and trans. PLoS genetics. 2015; 11(4):e1005136.

Carroll SB. Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. Cell. 2008; 134(1):25–36.

Chan YF, Marks ME, Jones FC, Villarreal Jr G, Shapiro MD, Brady SD, Southwick AM, Absher DM, Grimwood J, Schmutz J, et al. Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. science. 2010; 327(5963):302–305.

Gisselbrecht SS, Palagi A, Kurland JV, Rogers JM, Ozadam H, Zhan Y, Dekker J, Bulyk ML. Transcriptional silencers in Drosophila serve a dual role as transcriptional enhancers in alternate cellular contexts. Molecular cell. 2020; 77(2):324–337.

Halfon MS. Silencers, enhancers, and the multifunctional regulatory genome. Trends in Genetics. 2020; 36(3):149–151.

Howard ML, Davidson EH. cis-Regulatory control circuits in development. Developmental biology. 2004; 271(1):109–118.

Hughes JT, Williams ME, Johnson R, Grover S, Rebeiz M, Williams TM. Gene regulatory network homoplasy underlies recurrent sexually dimorphic fruit fly pigmentation. Frontiers in Ecology and Evolution. 2020; 8:80.

Jacob F, Monod J. Genetic regulatory mechanisms in the synthesis of proteins. Journal of molecular biology. 1961; 3(3):318–356.

Jeong S, Rokas A, Carroll SB. Regulation of body pigmentation by the Abdominal-B Hox protein and its gain and loss in Drosophila evolution. Cell. 2006; 125(7):1387–1399.

Johnson AD. The price of repression. Cell. 1995; 81(5):655–658.

Johnson WC, Ordway AJ, Watada M, Pruitt JN, Williams TM, Rebeiz M. Genetic changes to a transcriptional silencer element confers phenotypic diversity within and between Drosophila species. PLoS genetics. 2015; 11(6):e1005279.

Kassambara A. ggpubr: 'ggplot2' Based Publication Ready Plots; 2020, https://CRAN.R-project.org/package=ggpubr, r package version 0.4.0.

Lewis JJ, Geltman RC, Pollak PC, Rondem KE, Van Belleghem SM, Hubisz MJ, Munn PR, Zhang L, Benson C, Mazo-Vargas A, et al. Parallel evolution of ancient, pleiotropic enhancers underlies butterfly wing pattern mimicry. Proceedings of the National Academy of Sciences. 2019; 116(48):24174–24183.

Liu Y, Ramos-Womack M, Han C, Reilly P, Brackett KL, Rogers W, Williams TM, Andolfatto P, Stern DL, Rebeiz M. Changes throughout a genetic network mask the contribution of Hox gene evolution. Current Biology. 2019; 29(13):2157–2166.

Lopez-Rivera F, Foster Rhoades OK, Vincent BJ, Pym EC, Bragdon MD, Estrada J, DePace AH, Wunderlich Z. A mutation in the Drosophila melanogaster eve stripe 2 minimal enhancer is buffered by flanking sequences. G3: Genes, Genomes, Genetics. 2020; 10(12):4473–4482.

Markow TA, O'Grady P. Drosophila: a guide to species identification and use. Elsevier; 2005.

McGregor AP, Orgogozo V, Delon I, Zanet J, Srinivasan DG, Payre F, Stern DL. Morphological evolution through multiple cis-regulatory mutations at a single gene. Nature. 2007; 448(7153):587–590.

Ngan CY, Wong CH, Tjong H, Wang W, Goldfeder RL, Choi C, He H, Gong L, Lin J, Urban B, et al. Chromatin interaction analyses elucidate the roles of PRC2-bound silencers in mouse development. Nature genetics. 2020; 52(3):264–272.

Ordway AJ, Hancuch KN, Johnson W, Wiliams TM, Rebeiz M. The expansion of body coloration involves coordinated evolution in cis and trans within the pigmentation regulatory network of Drosophila prostipennis. Developmental biology. 2014; 392(2):431–440.

Pang B, Snyder MP. Systematic identification of silencers in human cells. Nature genetics. 2020; 52(3):254–263.

Payankaulam S, Li LM, Arnosti DN. Transcriptional repression: conserved and evolved features. Current biology. 2010; 20(17):R764–R771.

Prud'homme B, Gompel N, Rokas A, Kassner VA, Williams TM, Yeh SD, True JR, Carroll SB. Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. Nature. 2006; 440(7087):1050–1053.

R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria; 2022, https://www.R-project.org/.

Rebeiz M, Patel NH, Hinman VF. Unraveling the tangled skein: the evolution of transcriptional regulatory networks in development. Annual review of genomics and human genetics. 2015; 16:103.

Rebeiz M, Pool JE, Kassner VA, Aquadro CF, Carroll SB. Stepwise modification of a modular enhancer underlies adaptation in a Drosophila population. Science. 2009; 326(5960):1663–1667.

Rebeiz M, Williams TM. Experimental approaches to evaluate the contributions of candidate cis-regulatory mutations to phenotypic evolution. Molecular Methods for Evolutionary Genetics. 2012; p. 351–375.

Rebeiz M, Williams TM. Using Drosophila pigmentation traits to study the mechanisms of cis-regulatory evolution. Current Opinion in Insect Science. 2017; 19:1–7.

Segert JA, Gisselbrecht SS, Bulyk ML. Transcriptional silencers: Driving gene expression with the brakes on. Trends in Genetics. 2021; 37(6):514–527.

Sengupta AK, Kuhrs A, Muller J. General transcriptional silencing by a Polycomb response element in Drosophila. Development. 2004; .

Shashikant T, Ettensohn CA. Genome-wide analysis of chromatin accessibility using ATAC-seq. Methods in cell biology. 2019; 151:219–235.

Signor SA, Liu Y, Rebeiz M, Kopp A. Genetic convergence in the evolution of male-specific color patterns in Drosophila. Current Biology. 2016; 26(18):2423–2433.

Voutev R, Mann RS. Robust ΦC31-mediated genome engineering in Drosophila melanogaster using minimal attP/attB phage sites. G3: Genes, Genomes, Genetics. 2018; 8(5):1399–1402.

Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York; 2016. https://ggplot2.tidyverse.org.

Wittkopp PJ, Kalay G. Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nature Reviews Genetics. 2012; 13(1):59–69.

Wittkopp PJ, True JR, Carroll SB. Reciprocal functions of the Drosophila yellow and ebony proteins in the development and evolution of pigment patterns. Development. 2002; .

Wittkopp PJ, Vaccaro K, Carroll SB. Evolution of yellow gene regulation and pigmentation in Drosophila. Current Biology. 2002; 12(18):1547–1556.

Yokoshi M, Segawa K, Fukaya T. Visualizing the role of boundary elements in enhancer-promoter communication. Molecular cell. 2020; 78(2):224–235.

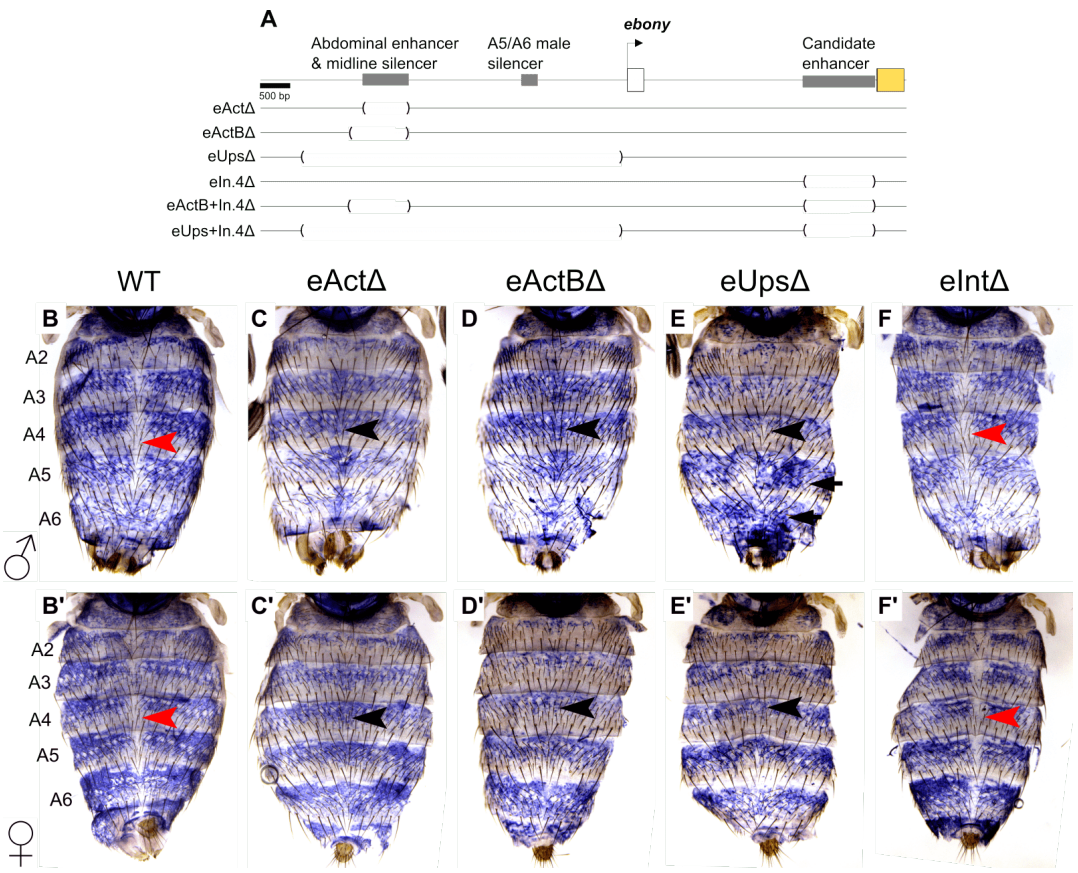

Figure 2–Figure supplement 1. (A) Gene map of the *ebony* locus showing the location of the deletions created to identify redundant enhancers. **(B-F')** *ebony* abdominal *mRNA* expression measured with *in-situ* hybridization in recently eclosed adults for WT, *ebony* null mutants, and deletion lines males and females

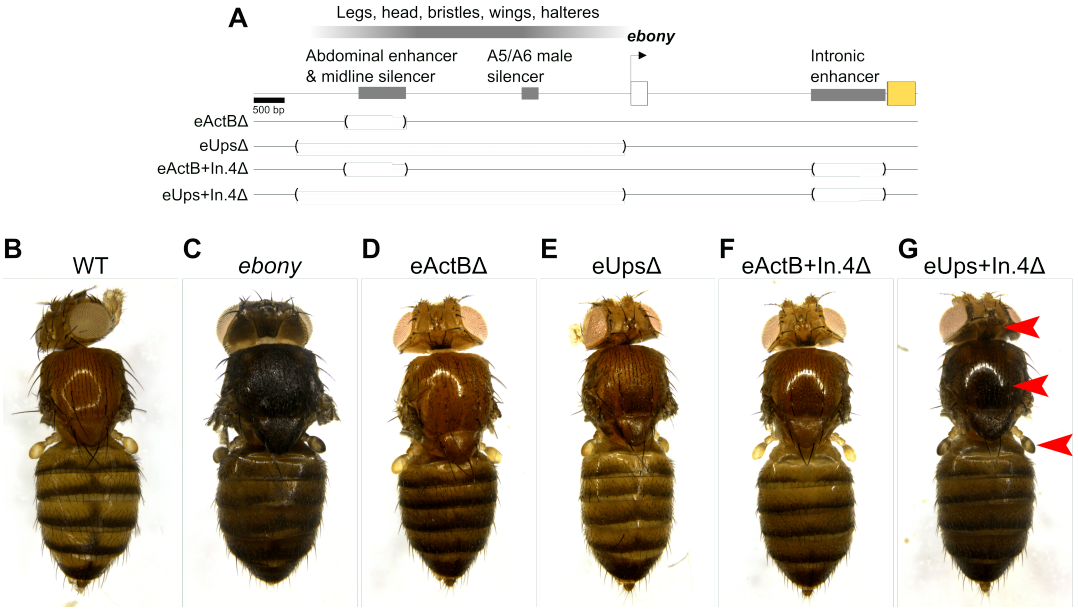

Figure 2–Figure supplement 2. **(A)** Gene map of the *ebony* locus showing the location of the deletions created to identify redundant enhancers. Previously identified tissue-specific enhancers are shown on top of the *ebony* upstream region (shaded rectangle). **(B-G)** Pigmentation of different adult tissues in females from the different strains created. Red arrows show tissues, other than the abdomen, with darker pigmentation compared to the WT and more similar to ebony mutants.

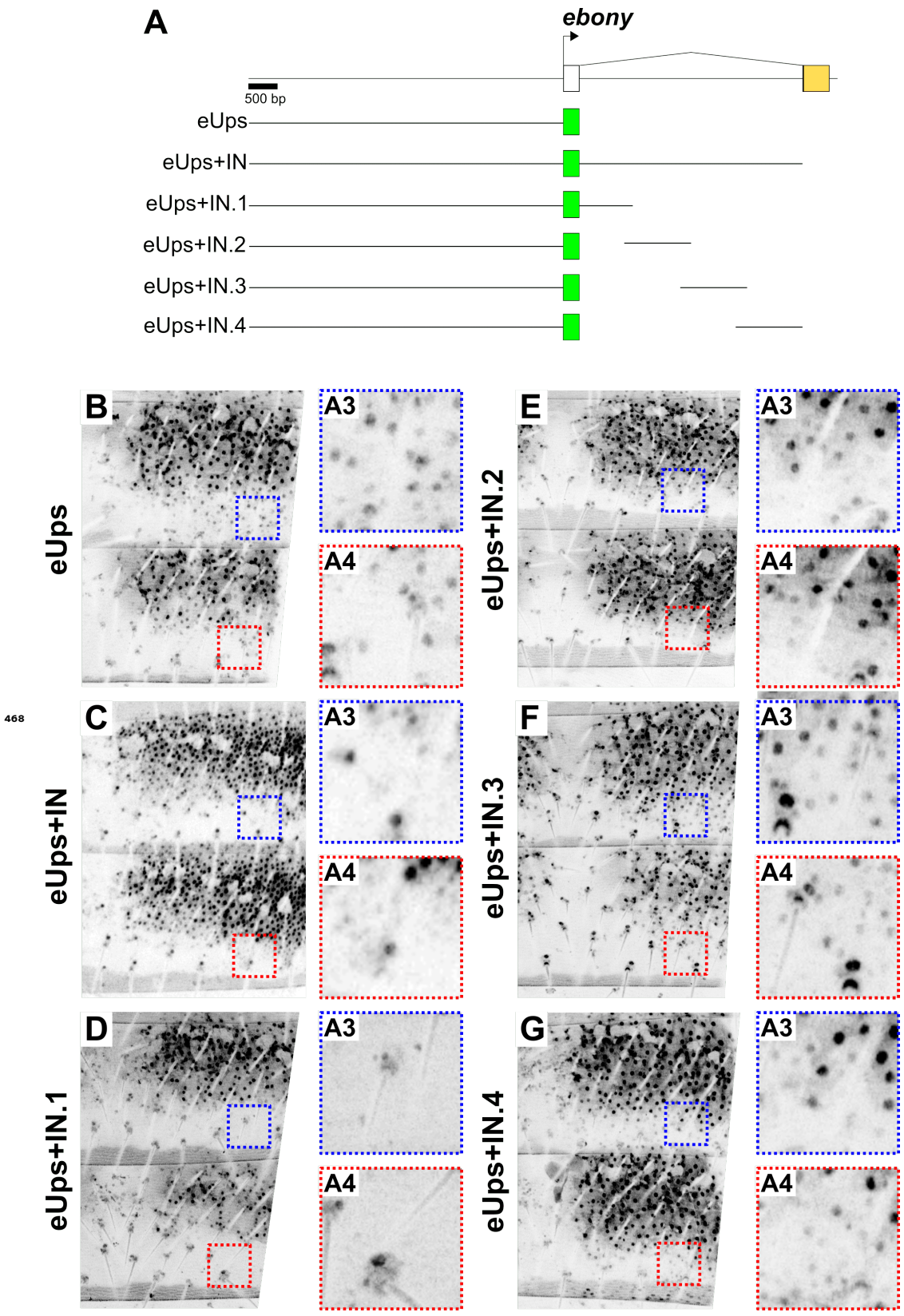

Figure 3–Figure supplement 1. **(A)** Gene map of the *ebony* locus showing the location of the reporter constructs created to identify the stripe silencer within the first intronic region. **(B-G)** GFP expression pattern of the different transgenic reporters at 24h after eclosion. Blue and red dashed boxes show a magnification of the stripe area in A3 and A4, respectively.

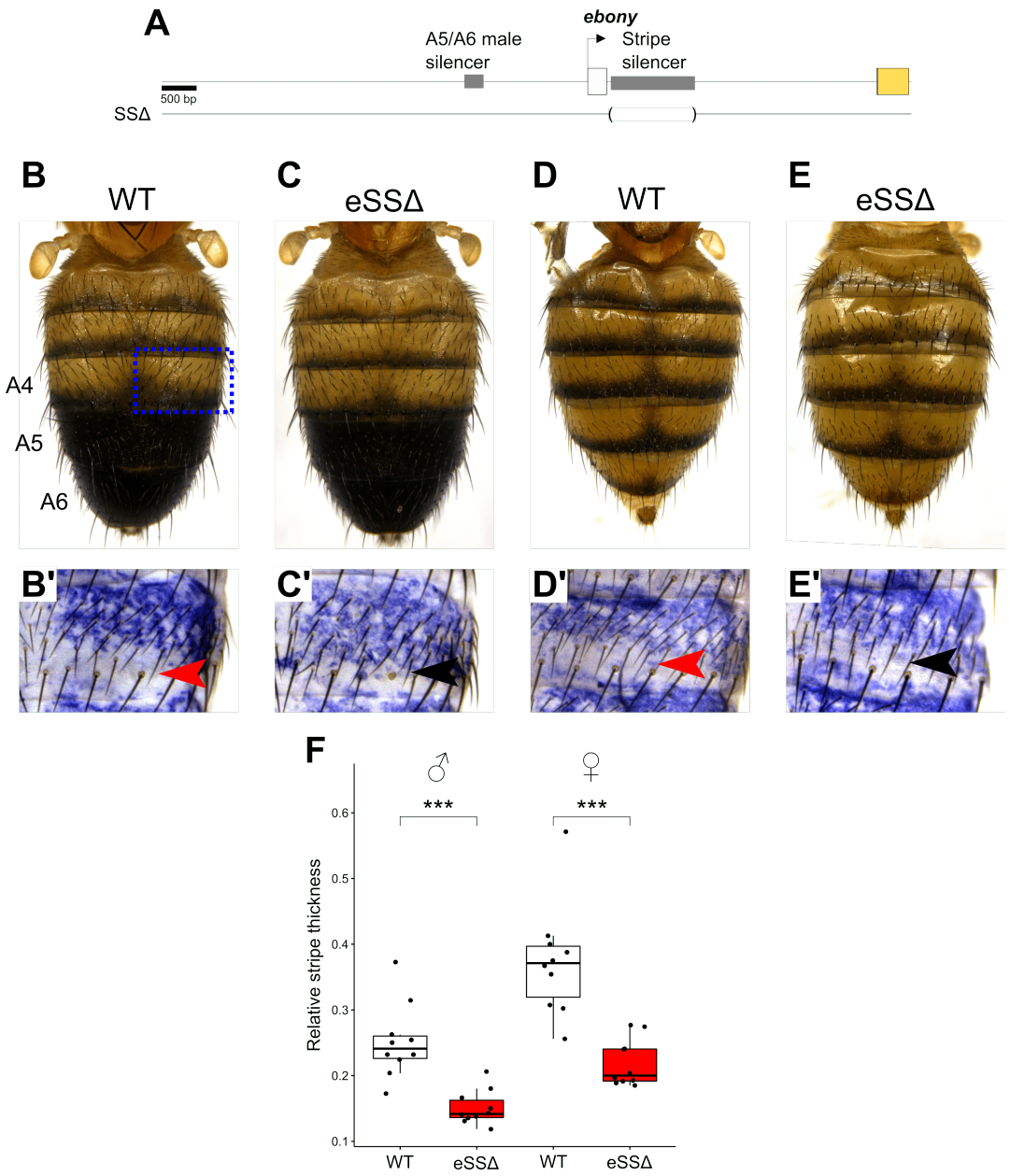

Figure 3–Figure supplement 2. **(A)** Gene map of the *ebony* locus showing the location of the deletion targeting the stripe silencer (*eSSΔ*). **(B-E)** Adult pigmentation of WT **(B)** and *eSSΔ***(C)** males and females. **(B'-E')** *In-situ* hybridization detecting *ebony mRNA* in the A4 segment of WT **(B')** and *eSSΔ*males and females. Red and black arrowheads indicate low and increased levels of *ebony mRNA*, respectively. **(F)** Comparison of the relative thickness of the melanic stripe between WT and *eSSΔ*males and females. (Student's t test, *** = $p < 0.0005$)

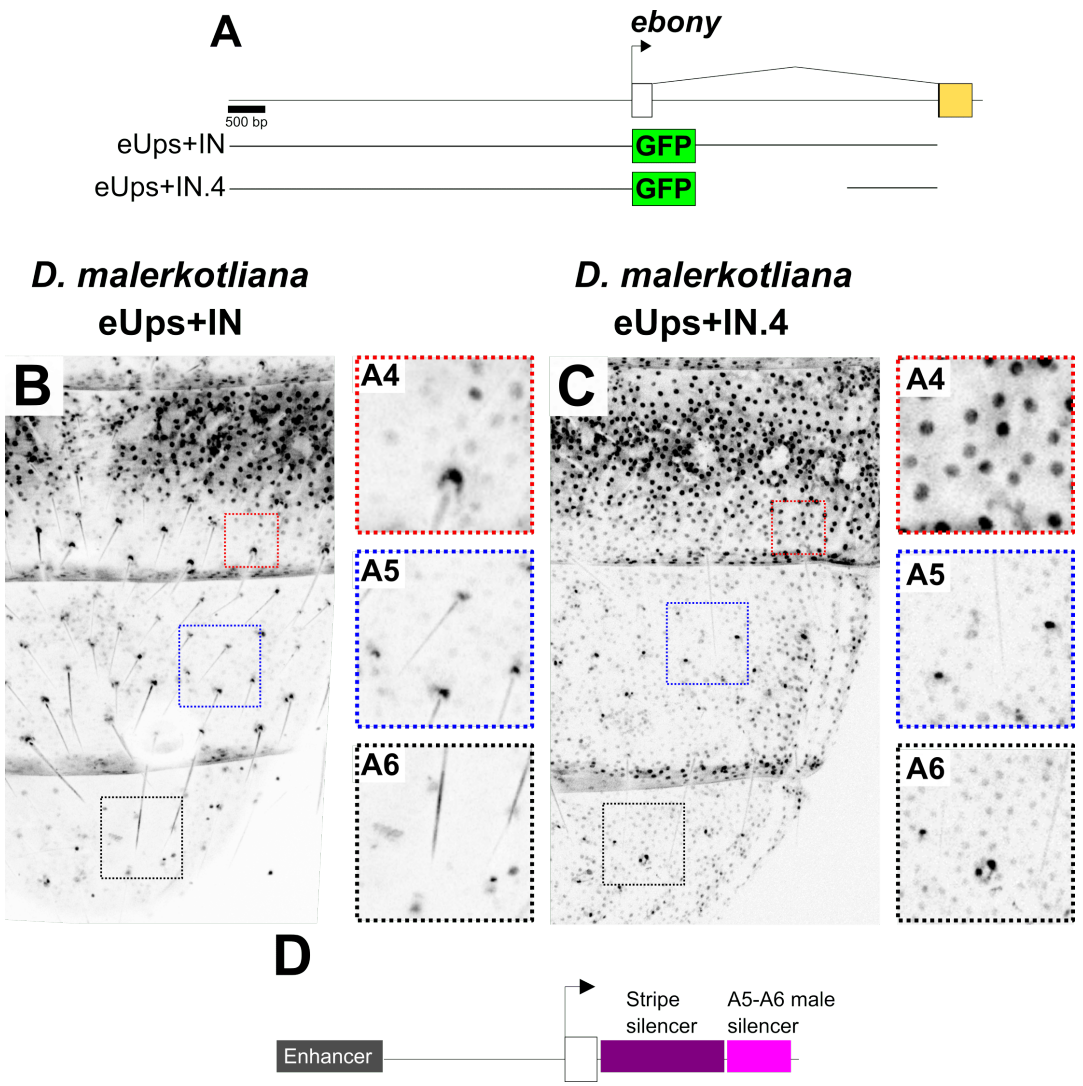

Figure 4–Figure supplement 1. (A) Gene map showing the reporter constructs created to identify the location of the *D. malerkotliana* male silencer within the first *ebony* intron. **(B-C)** GFP expression pattern of *D. malerkotliana* transgenic reporter eUps+IN and eUps+IN.4. Boxed regions show expression in A4 stripe region (red), and A5-A6 segments (blue and black, respectively). **(D)** Inferred location of the *D. malerkotliana* intronic silencer within the first *ebony* intron.

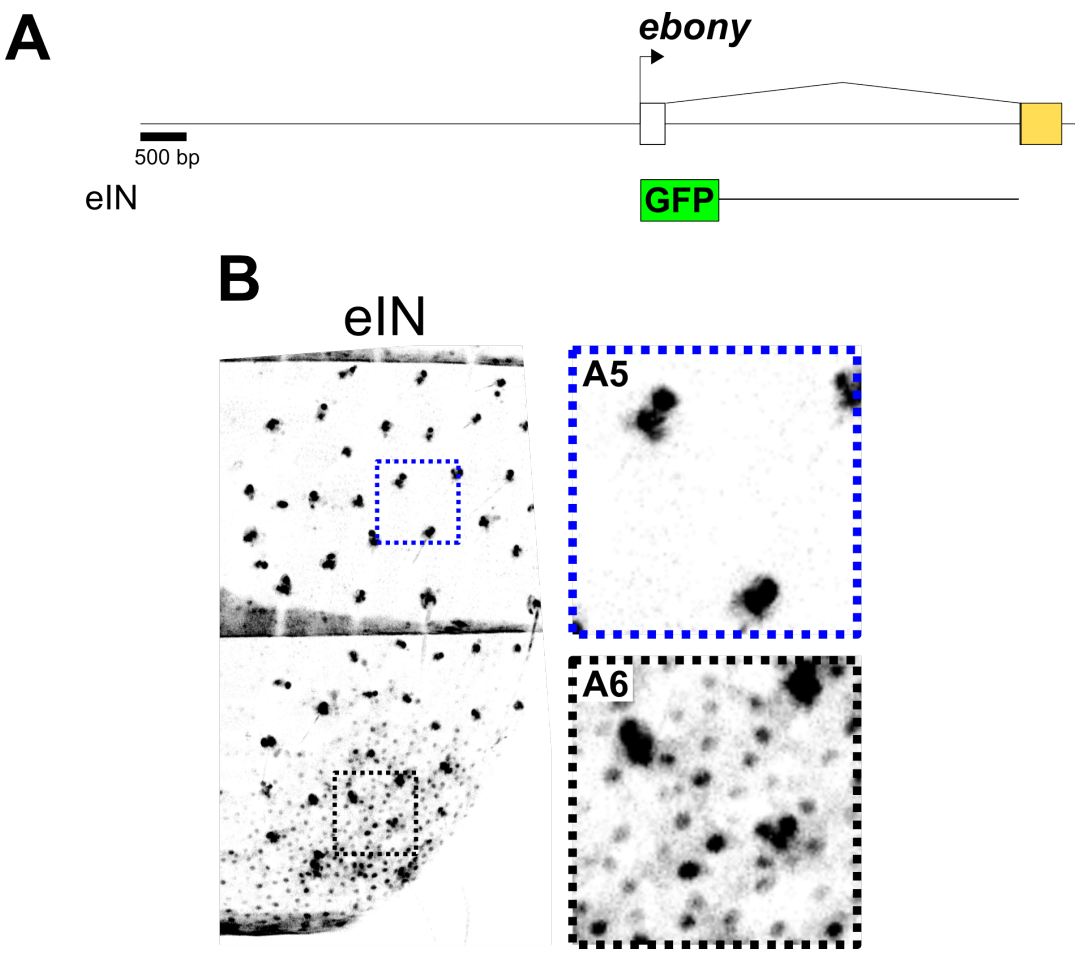

Figure 4–Figure supplement 2. **(A)** Gene map showing the reporter constructs created for *D. pseudoobscura*. **(B)** GFP expression patterns of *D. pseudoobscura* transgenic reporter eIN.

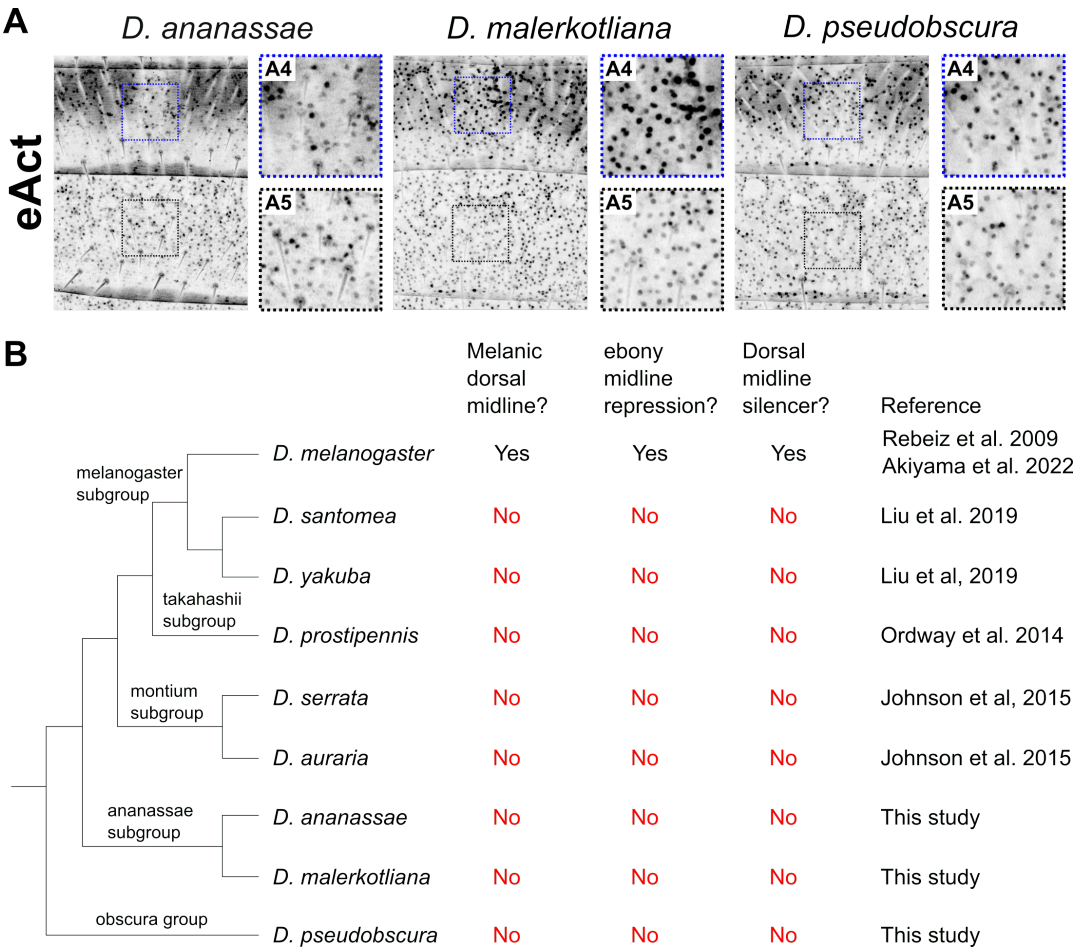

Figure 4–Figure supplement 3. (A) GFP expression patterns of the eAct transgenic reporters in the abdominal segments A4-A5. Insets show magnified regions along the midline for A4 (red square) and A5 (blue square). **(B)** Phylogenetic distribution of the melanic dorsal midline in *Drosophila* species for which the expression and regulation of *ebony* in this area has been studied.