Evolution of repressive and temporal regulatory elements underlies the diversity of *Drosophila* abdominal pigmentation

by

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A major question in evolutionary biology concerns the developmental origin of morphological diversity. Changes in gene regulation are known to contribute to developmental differences producing morphological variation. Interspecific divergence in gene expression often results from changes in transcription-stimulating enhancer elements. However, the role of other kinds of regulatory elements in this process has been less explored. In my doctoral work, I used the abdominal melanic pigmentation of *Drosophila* to analyze how changes in the function of distinct regulatory elements can contribute to morphological evolution. In Chapter 1, I investigated the contribution of repressive transcriptional silencers. I show that the *Drosophila* pigmentation gene *ebony* has mainly evolved through changes in the spatial domains of silencers that pattern its abdominal expression. By precisely editing the endogenous *ebony* locus of *D. melanogaster*, I demonstrate the requirement of two redundant abdominal enhancers and three silencers that repress the redundant enhancers in patterned manner. Then, I demonstrate that transcriptional silencers have been involved in every case of *ebony* evolution observed to date. These findings suggest that negative regulation by silencers likely has an under-appreciated role in gene regulatory evolution. In Chapter2, I studied the evolution of temporal regulation of the *Hox*-gene *Abd-B*. The expression of this gene during mid pupal development is crucial for the formation of male-specific abdominal melanic pigmentation. I show that this expression pattern evolved recently in *D. melanogaster* and closely related species.

I identified two temporally restricted *cis*-regulatory elements required for this expression pattern. Furthermore, using allele replacements, I show that the function of these CREs is highly conserved even in species lacking Abd-B expression. My results suggest that a novel expression pattern evolved using conserved CREs, and suggests the existence of changes in unknown silencers, and/or upstream factors. I propose that heterochronic shifts in Hox-gene expression might be a common mechanism to modify the morphology of animal segments while reducing pleiotropic effects. In summary, my work highlights the importance of repression and temporal regulation in the evolution of novel expression patterns and morphological traits.

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1.1 The genetic basis of morphological evolution

A major question in evolutionary biology concerns the origin of morphological traits. Because animal body parts are formed during development, it follows that morphological diversity results from alterations of developmental processes. Interestingly, animal development is controlled by a common set of conserved patterning genes [Carroll, 2008]. How can different morphological traits evolve using the same developmental genes? An important finding of evolutionary developmental biology is that morphological evolution largely results from changes in the expression (i.e., regulatory evolution) rather than in the function of developmental genes [Prud'homme et al., 2007]. In fact, genomic analysis suggest that more than 50% of genes show expression differences between closely related species of yeast [Tirosh et al., 2009] and fruit flies [McManus et al., 2010].

Because most developmental genes serve multiple roles (i.e., they are pleiotropic), mutations affecting protein function are unlikely to be advantageous in multiple contexts. By producing discrete tissue-specific expression changes, regulatory evolution is expected to reduce possible deleterious effects. Hence, interspecific morphological variation can result from the same gene being differentially expressed. Early evidence supporting this mode of evolution comes from comparisons of appendage morphology in crustaceans. In most arthropods, thoracic appendages are used for locomotor functions. In some crustaceans, however, limbs from the anterior thorax have evolved a feeding function. The differences in morphology and function correlate with the expression of the *Hox*-genes *Ubx-AbdA* which in many crustaceans are

restricted from the anterior appendages. Thus, the lack of expression of these genes in thoracic segments has been hypothesized to contribute to the evolution of feeding appendages [Averof and Patel, 1997].

While correlations between gene expression and morphological differences are often observed among species, identifying the mechanisms responsible for such expression differences can be challenging. Stern [1998] found a correlation between expression of the *Hox* gene *Ubx* and trichome number in different *Drosophila* species. The pattern of sequence conservation suggested that evolution has targeted putative *cis*-regulatory elements (CREs) of this gene, and not the protein itself. Hence, regulatory mutations seem to offer a flexible mechanism to deploy conserved genes in novel contexts resulting in morphological variation [Stern, 1998]. This concept is now central for evolutionary developmental biology and together with our refined knowledge about gene regulation has provided an increasingly detailed understanding of the genetic mechanisms that fuel morphological evolution.

1.2 Gene expression is controlled by *cis*-regulatory elements

A key element of animal development is the exquisite spatial and temporal control of gene expression [Halfon, 2020]. This regulation is carried out by different classes of interacting CREs. Most genes contain one or a few promoters located close to the transcription starting site. While promoters are required for transcription, they only produce basal levels of mRNA [Wittkopp and Kalay, 2012]. This expression is boosted by modular CREs known as 'enhancers', DNA sequences that are bound by a specific set of transcription factors and as a result increase transcription of a target gene in a specific spatiotemporal pattern. Most genes seem to be regulated

by multiple enhancers, each one typically controlling gene expression within a discrete spatial domain in a developing animal [Prud'homme et al., 2007]. However, enhancers can be active in multiple tissues or developmental stages (i.e., they are pleiotropic), or multiple enhancers can regulate the same expression pattern (i.e., they are redundant).

Traditionally, enhancers have been identified using gene reporters. In this approach, candidate sequences are tested for enhancer activity by cloning them upstream of a minimally active promoter driving expression of a reporter gene whose activity is easy to monitor, for example, a fluorescent protein [Halfon, 2019]. This construct can then be transformed in cells or whole organisms and the expression of the reporter gene can be monitored. If the reporter expression of a candidate enhancer region matches the endogenous expression of the target gene, this would suggest that the tested region is indeed an enhancer (**Fig. 1**). Using this approach, thousands of enhancers have been characterized. While this has greatly increase our knowledge about gene regulation, the focus on enhancers has resulted in few studies addressing the function of other kinds of CREs.

Just as important as positive regulation, but less well studied, negative gene regulation is crucial for gene expression. At least two classes of negative acting CREs have been described: i) transcriptional silencers, which repress expression of an otherwise active promoter in a specific spatiotemporal pattern [Halfon, 2020]; ii) Polycomb response elements (PREs), which recruit proteins of the Polycomb group (PcG) to mediate chromatin-based gene silencing [Müller and Kassis, 2006] (**Fig. 2B**). While not all genes are regulated by negative-acting CREs, silencing transcription to fine-tune gene expression is essential for animal development [Erceg et al., 2017].

During animal development, gene expression needs to be regulated across time

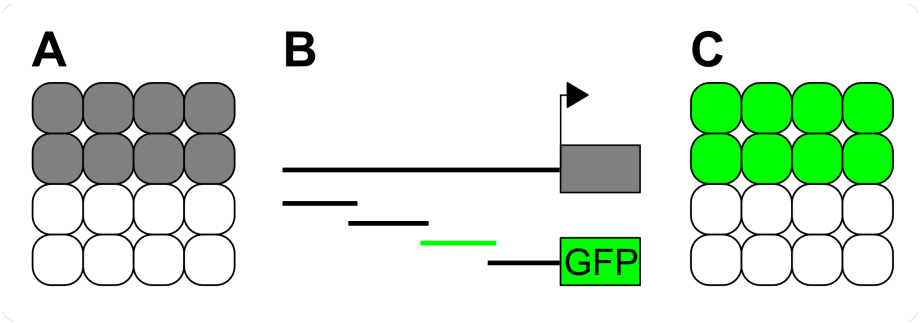

Figure 1: Identification of enhancers using gene reporter analysis. **A)** Hypothetical expression of a gene limited to the top two rows of cells (gray). **B)** Four fragments located upstream of this gene were tested for enhancer function using gene reporter analysis. Three of these regions did not drive reporter expression (black lines). **C)** One region drives reporter expression (green) in the top two rows of cells. Thus, this region is considered an enhancer of this gene.

and space. While some genes are only active during short windows of time, others may be active at different developmental stages. Most enhancers seem to contain the regulatory information to precisely regulate the spatiotemporal expression of their target genes [Small and Arnosti, 2020]. However, the regulation of *Hox* genes seem to be different. *Drosophila Hox*-genes contain two different classes of interacting enhancers: initiator elements, which initiate transcription of a target gene in a specific set of cells, and maintenance elements, which act later during development maintaining the expression state determined earlier on by an initiator element [Maeda and Karch, 2006]. In other words, the spatiotemporal regulation of these genes is controlled by interacting CREs, with initiator elements providing spatial information

and maintenance elements temporal information (**Fig. 2C**).

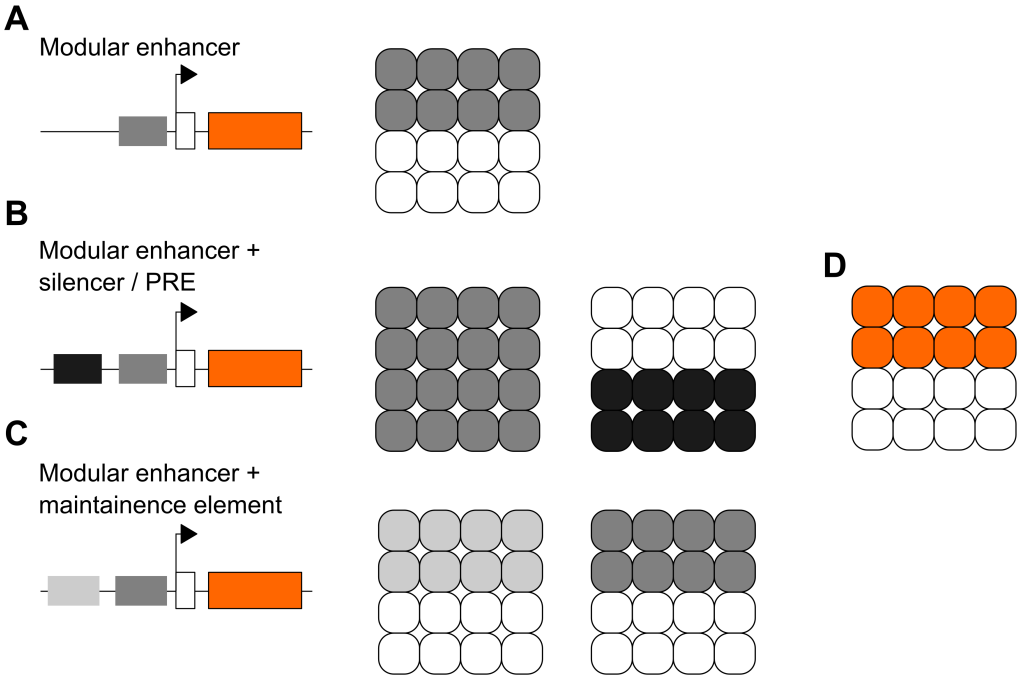

Figure 2: Gene expression is controlled by different classes of CREs. **A)** A modular enhancer (gray) activates expression of a gene (orange) exclusively in the two top rows of cells. **B)** A modular enhancer (gray) activates expression of a gene (orange) in all the cells, but this expression is repressed by a silencer/PRE (black) active in the two bottom rows of cells. **C)** A modular enhancer (light gray) initiates expression of a gene (orange) in the two top rows of cells, a maintenance element (gray) is required to maintain expression in these cells throughout development. **D)** In all cases, the final pattern of expression is the same with the gene being active in the two top rows of cells.

Since multiple classes of CREs can act on the same target gene, the interactions between these CREs ultimately determine the spatiotemporal expression pattern of

a such a gene (**Fig. 2D**). This has important implications for regulatory evolution since it suggests a high degree of flexibility for evolving new expression patterns. In principle any class of CREs could be the primary driver of regulatory evolution, however, most of the work has focused on enhancers. A key assumption of this approach, often experimentally confirmed, is that a unique subset of a gene's expression is controlled by a specific modular enhancer. Thus, new expression patterns and phenotypes could evolve in one part of the body, independent of other parts [Prud'homme et al., 2007], by the gain, inactivation, or modification of enhancer function. This approach has provided robust empirical evidence for the importance of CREs divergence and morphological evolution. However, more studies are needed to test whether regulatory evolution also occurs by targeting other classes of CREs, how often this happens, and in which context this mode of regulatory evolution is favored.

1.3 Morphological evolution by changes in the function of modular enhancers

Divergence in gene expression is widespread even between closely related species. However, the genetic basis of this divergent expression remains unknown for the vast majority of genes [Wittkopp and Kalay, 2012]. This is because elucidating the molecular mechanisms that are responsible for regulatory divergence is a complex empirical task that requires identifying the CRE (or CREs) that controls the expression pattern of interest and finding functionally divergent sites within this region that can explain the observed functional differences [Wittkopp and Kalay, 2012]. In the next sections I will review exceptional examples of morphological evolution resulting

from gains, losses and modification of modular enhancers.

1.3.1 Gain of modular enhancers

Novel morphological traits are often the result of evolutionary gains of enhancer function. In *Drosophila* species from the *melanogaster* lineage the posterior segments of the male abdomen are covered with melanic pigmentation. Crucial for this trait is the expression of the *yellow* gene in a pattern that mirrors the melanic pigmentation [Wittkopp et al., 2002b]. Jeong et al. [2006] found that this expression pattern is controlled by a *yellow* CRE that evolved specifically in the *melanogaster* lineage [Jeong et al., 2006]. Hence, a novel pigmentation trait coincides with the gain of a CRE, which spatial domain of activity evolved by acquiring novel regulatory inputs (**Fig. 3**). To understand how differences in enhancer function evolve at a microevolutionary scale, Wooldridge et al. [2022] studied the pigmentation of beach mouse subspecies. Two pigmentation morphs have been described, a light coloration morph found in the coast and that evolved multiple times from a mainland dark-colored morph ancestor. Population genetics analysis identified a highly differentiated DNA region fixed in lightly pigmented populations and found at low frequencies in mainland populations. This region contains an enhancer located close to the pigmentation gene *Agouti*, which promotes light pigmentation. Hence, evolutionary forces operating at the population level can act on regulatory alleles promoting the recurrent evolution of an adaptive trait [Wooldridge et al., 2022]. Together, these studies show how the gain of enhancer function contribute to the evolution of novel traits at different evolutionary scales.

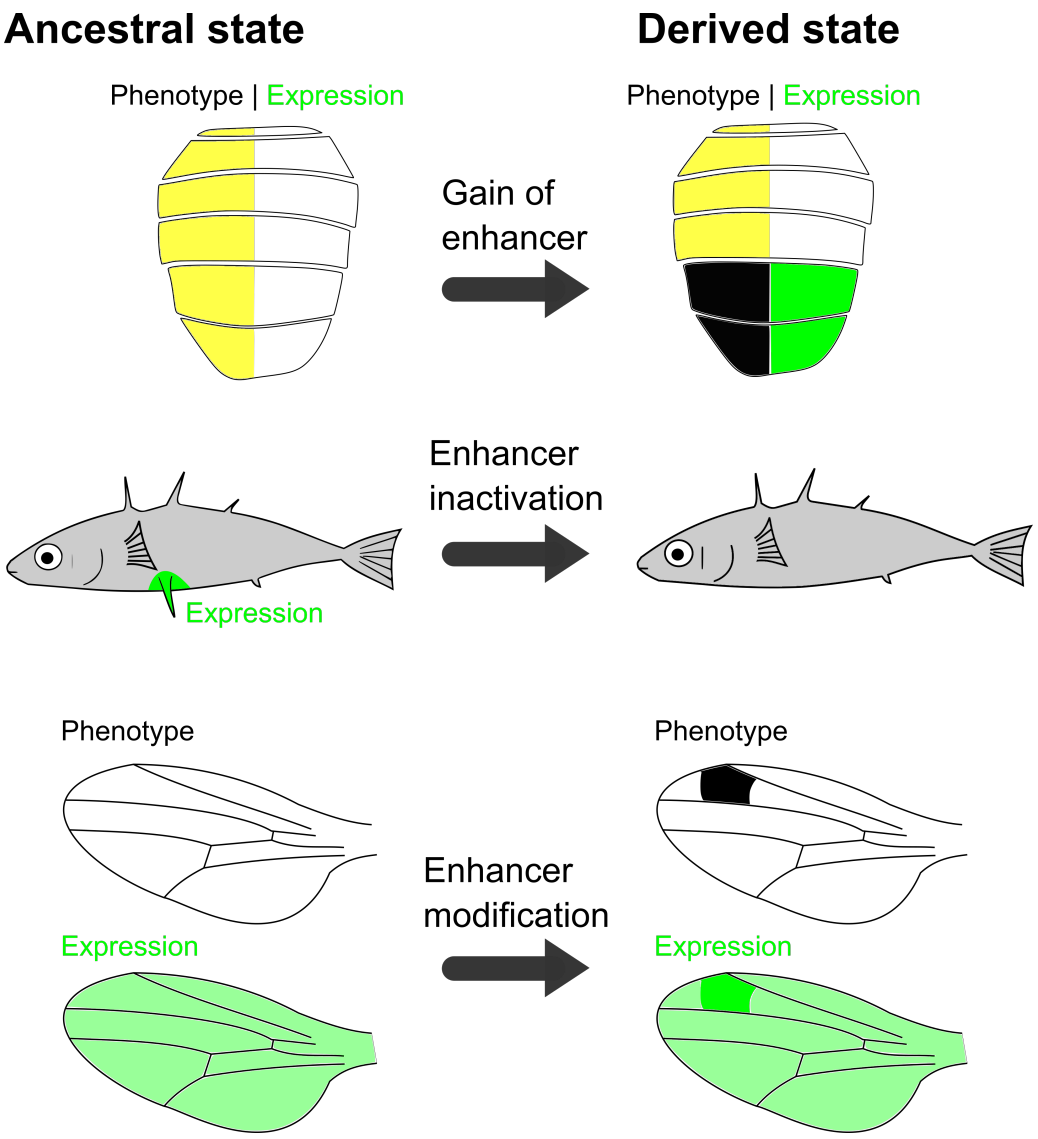

Figure 3: (Top) Evolution of a novel *yellow* enhancer contributed to the evolution of abdominal melanic pigmentation in *Drosophila*. (Middle) Inactivation of a *Pitx1* enhancer resulted in the loss pelvic spines in sticklebacks. (Bottom) Modification of a preexisting *yellow* enhancer contributed to the evolution of wing spots in *Drosophila*.

1.3.2 Inactivation of modular enhancers

The loss of morphological traits is a recurrent theme in morphological evolution. In fact, the frequency of trait losses is generally much larger than that of trait gains [Prud'homme et al., 2007]. A classic example of a major morphological change that evolved through loss is the limbless body plan of snakes. In vertebrates, a highly conserved enhancer is required for the expression of the gene *Sonic Hedgehog* (*Shh*) during limb development. This enhancer has been inactivated in snake lineages that completely lack vestigial hindlimbs [Kvon et al., 2016]. Thus, mutations inactivating the function of a key developmental enhancer seem to have contributed to a major morphological change during snake evolution.

Another example of morphological change by secondary losses is the repeated loss of pelvic spines in sticklebacks. These spines cover the ventral and lateral sides of fish from marine populations, and have been lost multiple times in freshwater populations. The secondary loss of pelvic spines correlates with the absence of *Pitx1* pelvic expression, a gene required for normal hindlimb development in vertebrates [Shapiro et al., 2004]. Chan et al. [2010] identified an enhancer active in the developing pelvic bud of marine sticklebacks which has lost function in freshwater populations [Chan et al., 2010]. Importantly, *Pitx1* null mutations are lethal, and thus tissue-specific loss of expression, through enhancer inactivation, may have represented the only viable path to evolve the spinless phenotype (**Fig. 3**).

1.3.3 Modification of enhancer function

Divergence in gene expression can result from the functional modification of a pre-existing enhancer. In many *Drosophila* species the wings of males are adorned with a characteristic spot of dark pigmentation. This trait has a complex evolutionary

history involving multiple independent gains and losses [Prud'homme et al., 2006]. However, in all species tested, the wing spot correlates with the wing expression of the pigmentation gene *yellow*. In non-spotted species, a conserved enhancer drives low *yellow* expression throughout the wing. Mutations affecting the function of this enhancer resulted in a spot-specific activity which seems to have been crucial for the evolution of this trait [Gompel et al., 2005]. Interestingly, the independent evolution of wing spots in one lineage involved the modification of a different *yellow* enhancer. These studies demonstrate how comparable phenotypes can evolve indepdently by mutations affecting the function of different enhancers controlling the expression of the same gene (**Fig. 3**).

As shown in the example above, the evolution of gene expression patterns can occur by targeting different enhancers. This suggests a high degree of evolutionary flexibility, however, it is not always clear what are the causal mutations that have evolved in a particular enhancer. To understand the number, effect, and order of mutations that alter enhancer function,Rebeiz et al. [2009] studied the regulatory evolution of the pigmentation gene *ebony* in a *Drosophila* population. The authors identified an important *ebony* enhancer in which at least five mutations with varied effects have modified its function. These mutations arose from a combination of standing variation and *de novo* mutations [Rebeiz et al., 2009]. Thus, this study was able to recreate the mutational pathway that led to the evolution of a regulatory allele with a large phenotypic effect.

1.4 Regulatory evolution beyond modular enhancers

As explained in the previous sections, changes in enhancer function are key for morphological evolution. Because multiple enhancers can control different expression patterns of the same gene, this provides a flexible mechanism for evolution to occur. However, the regulatory genome is more than just modular enhancers. In the next sections I will discuss the potential of two classes of CREs, transcriptional silencers and maintenance CREs, to fuel differences in gene expression.

1.4.1 Negative regulation through transcriptional silencers

Silencers are the repressive counterparts of enhancers, regulatory DNA elements that repress transcription of their target genes [Segert et al., 2021]. Like enhancers, silencers provide binding sites that recruit regulatory factors, in this case transcriptional repressors [Ogbourne and Antalis, 1998], and are active in a specific spatiotemporal manner which is retained when removed from their native genomic context [Zheng et al., 2004, Qi et al., 2015]. Although discovered decades ago [Brand et al., 1985], silencers have been much less studied than enhancers. Genome wide studies of silencers, however, suggest that these CREs are a pervasive feature of animal genomes [Gisselbrecht et al., 2020, Ngan et al., 2020, Pang and Snyder, 2020]. Thus, it is possible to hypothesize that similarly to enhancers, changes in silencer function might be a key process for morphological evolution.

Only a handful of studies has directly assessed the role of silencers in morphological evolution. Johnson et al. [2015] studied the regulatory evolution of the pigmentation gene *ebony* in different *Drosophila* species. In males, this gene is repressed from the most posterior abdominal segments by a male-specific silencer [Rebeiz et al.,

2009]. In some species, this silencer has been inactivated, which resulted in the up-regulation of *ebony* followed by a change in abdominal pigmentation [Johnson et al., 2015]. Another example is the wing pigmentation of *Heliconius* butterflies. In the wings of these species, the gene *Optix* directs the formation of red pigmentation. The complex expression pattern of this gene is regulated by multiple CREs. Using genome editing, Lewis et al. [2019] deleted these CREs and, as expected, found a decrease in red pigmentation. However, the deletion of a particular CRE resulted in the expansion of red patterns, suggesting that this region functions as a silencer [Lewis et al., 2019]. While it remains to be tested whether differences in the function of this silencer exist, it is possible that the diversity in red pigmentation patterns might have evolved from changes in both the function of enhancers and silencers.

These studies suggest that silencers can contribute to the evolution of morphological variation (**Fig. 4**). However, more studies are needed to determine the relative contribution of silencers vs enhancers in regulatory evolution, and identifying the context(s) in which negative regulation may be the preferred path for driving morphological evolution.

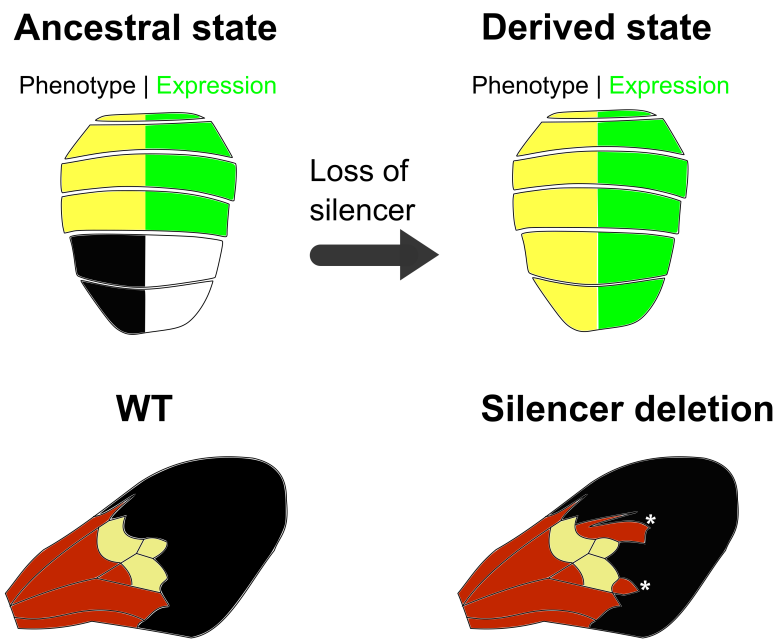

Figure 4: (Top) Loss of an *ebony* silencer contributed to the evolution of abdominal melanic pigmentation in *Drosophila*. (Bottom) Deletion of an *Optix* CRE resulted in the expansion of red pigmentation in *Heliconius* wings (asterisk) thus revealing a silencer function.

1.4.2 Temporal regulation of *Hox*-genes requires complex interactions between *cis*-regulatory elements

Hox-genes are a family of conserved transcription factors that are expressed in a segment-specific manner during animal development [Hughes and Kaufman, 2002]. Mutations affecting the function or expression of these genes produce "homeotic" transformations, the modification of a body segment's identity into that of another

segment [Lewis, 1978]. In *Drosophila*, each *Hox*-gene is active in a specific segment at multiple developmental stages. The regulation of these genes represent an outstanding example of how multiple classes of CREs are required to produce a specific spatiotemporal pattern of expression.

In *Drosophila*, the *Hox*-genes patterning the most posterior segments and their regulatory regions are located in the Bithorax complex [Duncan, 1987]. This complex contains three genes (*Ubx, abd-A,* and *Abd-B*) and multiple large regulatory regions. The most posterior *Hox*-gene active in the abdominal segments A5, A6, A7 and A8 is *Abd-B*. The segment-specific expression of this gene is controlled by the *infra-abdominal* (*iab*) regulatory domains. For example, in A5, *Abd-B* is regulated by the *iab-5* domain, while *iab-6* does the same in A6 [Celniker et al., 1989]. The function of the *iab* regions relies on interactions among different classes of CREs. Each *iab* region contains an initiator element that is bound by embryonic transcription factors (TFs) and as a result sets the activity state (ON/OFF) of the domain early in embryogenesis [Maeda and Karch, 2006]. The ON state is maintained by Trithorax group proteins, while the OFF state is maintained by Polycomb group proteins which are recruited to Polycomb Responsive Elements (PRE) [Busturia and Bienz, 1993]. Active *iab* domains can drive tissue specific expression during later developmental stages through maintenance enhancers (mCREs) which are bound by different sets of TFs. The function of these mCREs depends on the activation of the *iab* domain they are part of [Iampietro et al., 2010]. Thus, their spatial domain of activity is not intrinsic to them, but determined by their endogenous context. Finally, each *iab* domain is flanked by boundary elements which block enhancer-promoter interactions preventing the wrong *iab* region to be active in wrong segment (**Fig. 5**) [Maeda and Karch, 2006].

Comparative work suggests that the *iab-5* initiator element is functionally con-

served among different *Drosophila* species despite relatively low levels of sequence conservation [Mihaly et al., 2006]. Less is known, however, about the number, location, and evolution of *iab* mCREs. Recently, three putative *iab-5* mCREs were identified in *D. melanogaster* [Postika et al., 2021]. However, the specific temporal activity of these regions has not been addressed. Thus, it is unclear whether these elements are functionally redundant or if each mCRE is active in a specific developmental stage. Understanding the temporal regulation of *Hox*-genes is crucial to inform our models about the evolution of *Hox*-regulated traits. For example, *Abd-B* regulates traits that form at different developmental stages, like larval neurons, bristles, trichomes, and melanic pigmentation [Kopp et al., 2000]. If different mCREs control *Hox*-genes expression at specific developmental stages, then functional changes affecting a specific mCRE would only affect the development of some traits, thus reducing potential deleterious effects. Liu et al. [2019] provided evidence of this mode of regulatory evolution by studying the evolution of abdominal melanic pigmentation between the sister species *D. yakuba* and *D. santomea*. While *Abd-B* expression during early development is conserved between the two species, *D. santomea* lacks *Abd-B* expression during late pupal development, which is necessary for the formation of melanic pigmentation. This temporal difference in expression is explained by changes in the function of putative *iab-5* mCREs [Liu et al., 2019]. Thus, the diversification of *Hox*-regulated traits may, sometimes, be the result of temporal-specific changes in expression through the modification of mCREs.

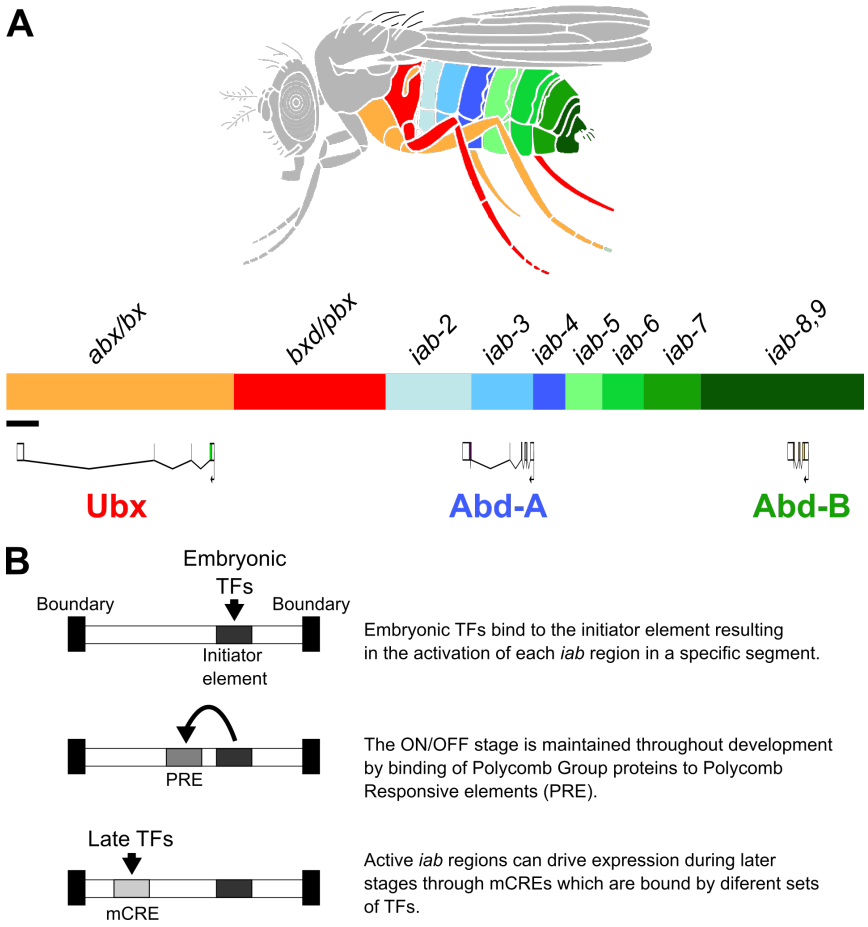

Figure 5: **A)** Diagram of the Bithorax complex. The multicolored bar represents the DNA of the BX-C. Individual *cis*-regulatory domains are indicated by the different colored regions on this bar. Orange/red, blue, and green regions control *Ubx*, *abd-A*, and *Abd-B* expression, respectively. The corresponding adult segments affected by mutations in each *cis*-regulatory domain are indicated on the diagram of the adult fly using the same color code. Modified from Maeda and Karch [2006]. **B)** Basic mechanism of function of *iab* regulatory domains.

1.5 Addressing the endogenous function of *cis*-regulatory elements using genome editing

Divergence in the function of enhancers can be studied using transgenic reporter assays. In this experiment, the regulatory function of orthologous sequences from different species is analyzed using gene reporters. These constructs can be introduced in the genome of a test strain which allows for comparison of reporter expression in an identical genetic background. However, this approach may be limited when studying CREs which function relies on interactions (often occurring over long distances) with other regulatory elements. Likewise, while it is possible to identify silencers using reporters (by combining an enhancer with a putative silencer in the same construct), this requires the identification of an enhancer in the first place which can be time-consuming and technically challenging [Rebeiz et al., 2009]. This is similar for mCREs, which function depend on the interaction with initiator elements. In principle, it is possible to address the function of mCREs by combining them with their initiator element in a single reporter construct, but this is only possible if the latter is already known.

An alternative and complementary approach to gene reporter analysis is the deletion of endogenous CREs using CRISPR-Cas9. Endogenous deletions can be used to confirm the function of CREs identified using gene reporters. For instance, if the deletion of an enhancer results in the loss of expression of its target gene, then this indicates that this enhancer is necessary and sufficient for that specific expression pattern (**Fig. 6A**). However, if the expression is not affected after the enhancer deletion, this could indicate the presence of other enhancers with the same function (i.e., redundant enhancers) (**Fig. 6B**) [Hong et al., 2008]. Likewise, if a deletion increases the expression of a gene or is now expressed in a tissue where it was

not normally expressed (i.e., derepressed), this would indicate that the deleted region has a silencer function (**Fig. 6C**). Endogenous deletions offer a powerful method to study mCREs [Iampietro et al., 2010]. By deleting candidate regions and then assessing gene expression at specific developmental stages, it is possible to identify mCREs without previous knowledge of the interactions needed for the function of this region (**Fig. 6D**).

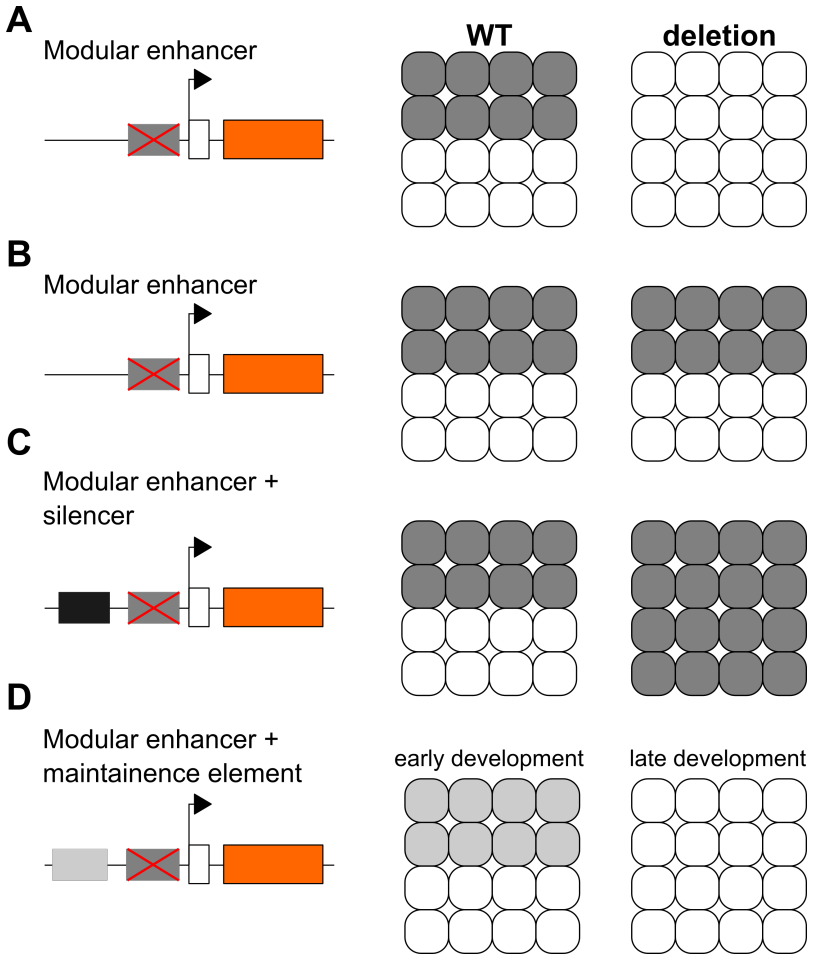

Figure 6: Using endogenous deletions to study different classes of CREs. **A)** Deletion of a modular enhancer results in the loss of expression. **B)** Deletion of a modular enhancer has no effect on the expression pattern, suggesting the presence of redundant enhancers. **C)** Deletion of a silencer results in gene expression outside its normal pattern (i.e., derepression). **D)** Deletion of a mCRE results in the loss of expression only during late development.

Ultimately, regulatory evolution aims to provide evidence that a candidate CRE

harbors variation that causes phenotypic differences. Experimental evidence for this could be obtained by replacing a CRE from species 'A' with the orthologous region from species 'B' and then evaluating the resultant phenotypes. If this CRE is the causal difference between these two species, then this replacement should result in the phenotypic modification of species 'A' into species 'B'. While this approach is considered the 'gold standard' in the field [Stern, 2014], the requirement of refined genome editing tools makes it feasible in only a few species. Auradkar et al. [2021] replaced the entire proboscipedia *Hox*-gene of *D. melanogaster*, which controls the development of the mouth parts, with that from *D. mimica*, a related species with highly modified mouth parts. While some aspects the adult morphology remained unchanged, others were modified in a way that resembled *D. mimica* morphology. Thus, this experiment showed that changes in the regulation of the *Hox*-gene proboscipedia are the cause of morphological differences between these two species [Auradkar et al., 2021]. Altogether, genome editing technologies such as CRISPR-Cas9 offer a powerful tool to causally link functional divergence in regulatory regions with morphological evolution.

1.6 *Drosophila* abdominal pigmentation as a model to study regulatory evolution

Drosophila abdominal pigmentation represents an ideal model to study regulatory evolution for three main reasons [Rebeiz and Williams, 2017]. First, it is a highly diverse trait with variation existing both within and between species. Second, the development of this trait is well understood, and multiple genes and their CREs have been characterized. Third, the presence of this trait in *D. melanogaster*

makes it possible to study it empirically using genetic manipulation of genes and their CREs. Abdominal pigmentation develops during late pupal stages by the production of three main pigments, black melanin, brown melanin, and yellow sclerotin [Wittkopp et al., 2002a]. In males of *D. melanogaster*, the dark pigmentation covering the most posterior dorsal cuticular plates (abdominal segments 5 and 6, A5-A6), is regulated by the *Hox*-gene *Abdominal-B* (*Abd-B*) [Celniker et al., 1989, Kopp et al., 2000] (**Fig. 7A**). Abd-B directly and indirectly activates the transcription of two genes promoting dark pigmentation, *yellow* [Jeong et al., 2006] and *tan* [Camino et al., 2015], respectively . Likewise, Abd-B contributes to the repression of the transcription factor *bab* [Williams et al., 2008] and the pigmentation gene *ebony*, both involved in the formation of light pigmentation (**Fig. 7B**). Thus, Abd-B represents a crucial regulator of A5-A6 melanic pigmentation in *D. melanogaster*.

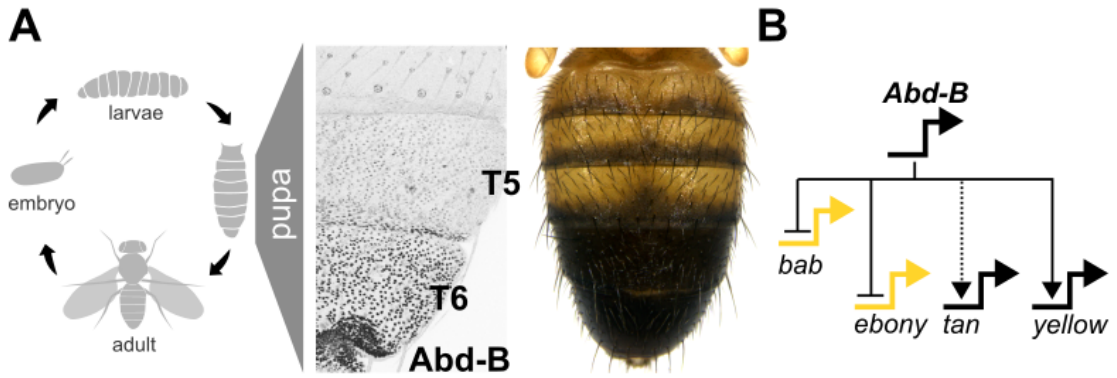

Figure 7: Development of *Drosophila* abdominal pigmentation **A)** In *D. melanogaster*, the *Hox*-gene *Abd-B* is expressed during pupal stages in A5 and A6, where it controls the formation of melanic pigmentation. **B)** *Abd-B* activates the pigmentation genes *yellow* (directly) and *tan* (indirectly), and it represses the yellow pigment promoting genes *bab* and *ebony*.

Ancestral character reconstruction analysis suggests that A5-A6 melanic pigmentation evolved from a non-melanic ancestor after the divergence of the *willistoni* and *melanogaster* species groups [Jeong et al., 2006] (**Fig. 8**). Within the *melanogaster* lineage, *bab*, *yellow*, and *tan*, evolved to be regulated by Abd-B which seems to have been crucial for the evolution of A5-A6 melanic pigmentation [Williams et al., 2008, Jeong et al., 2006, Camino et al., 2015]. The secondary losses observed in different species in this lineage (**Fig. 8**) involved changes in the enhancers of pigmentation genes like *yellow* and *tan*, as well as upstream regulators [Jeong et al., 2006, Camino et al., 2015, Hughes et al., 2020]. Likewise, the loss of transcriptional silencers regulating *ebony* has been linked with the loss of melanic pigmentation in some species [Ordway et al., 2014, Johnson et al., 2015, Liu et al., 2019]. In chapter two, I show

how the divergence in the spatial activity of *ebony* silencers has also contributed to the variation in abdominal melanic pigmentation. Moreover, by creating endogenous deletions, I analyzed the interaction between silencers and redundant enhancers.

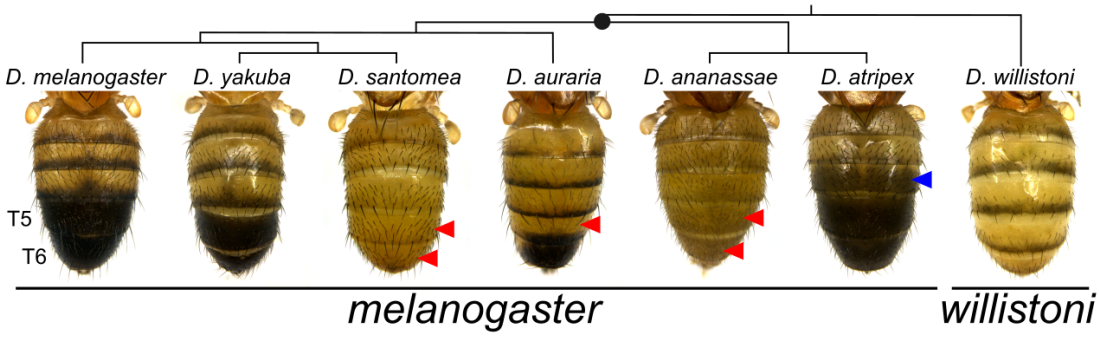

Figure 8: Male abdomens of *Drosophila* species from different lineages. The black circle indicates the split between the *melanogaster* and *willistoni* species groups. Red and blue arrows indicate secondary losses and expansions of melanic pigmentation, respectively.

As mentioned before, Abd-B is a major regulator of A5-A6 melanic pigmentation. Technical limitations, however, have limited our knowledge about the regulatory evolution of this gene. Liu et al. [2019] found that the non-melanic pigmentation of *D. santomea* involved the loss of Abd-B A5 expression. Since the enhancers of *yellow* and *tan* have lost their responsiveness to *Abd-B*, restoring Abd-B A5 expression does not affect the pigmentation in this species. Thus, the genetic program regulating the development of abdominal pigmentation has been extensively remodeled in *D. santomea*. Whether Abd-B expression changes have also ocurred in other species remains to be tested. In chapter three, I show that Abd-B expression differences during late pupal stages have taken place in other lineages. Likewise, I identified the

regulatory regions mediating these expression differences and their contribution to the evolutionary gain of A5 pigmentation.

Altogether, the results presented here suggest that regulatory evolution is not limited to changes in the function of modular enhancers, and highlight the importance of other kinds of CREs. Hence, differences in the function of repressive and temporal regulatory elements can contribute to variation in gene expression and, ultimately, to morphological evolution.

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

2.1 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 (**Fig. 9A**) [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 (**Fig. 9B**) 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, I investigated the *cis*-regulatory evolution of *ebony* in *D. melanogaster* and relatives displaying a range of pigmentation phenotypes (**Fig. 9B**). I 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. I 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 re-

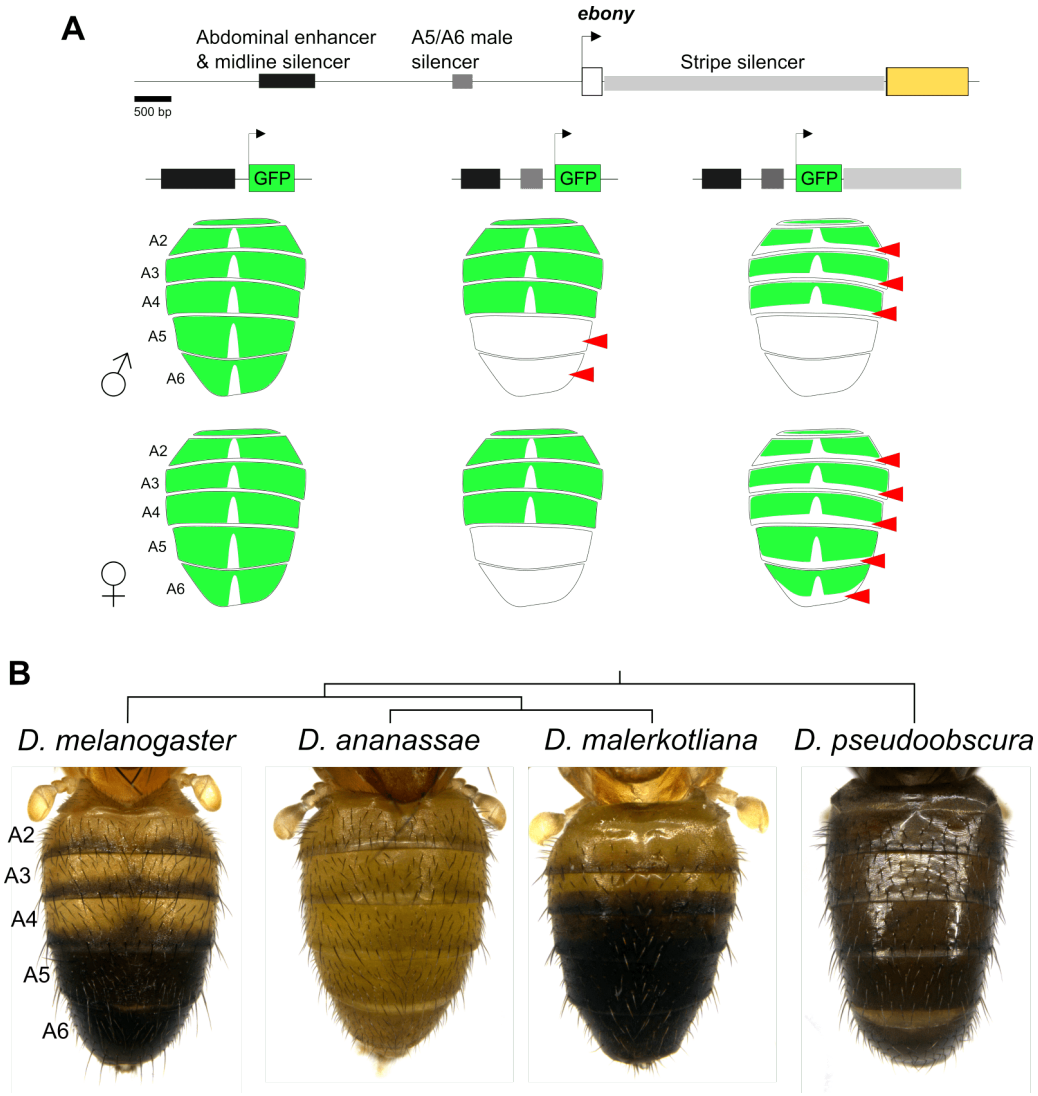

Figure 9: *ebony* abdominal expression is patterned by multiple regulatory elements. **(A)** Gene map of the *ebony* locus showing the location of known enhancers and transcriptional silencers active in the abdomen of *D. melanogaster*. The cartoons below represent the GFP reporter expression of the upstream enhancer alone and in combination with the two silencers. **(B)** Phylogeny showing the abdominal pigmentation of males from different *Drosophila* species.

sulting from changes in the function of transcriptional silencers can contribute to phenotypic diversity.

2.2 Results

2.2.1 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]. However, the number and location of such enhancers has not been determined. I used CRISPR-*Cas9* to create a series of deletions aiming to identify the redundant enhancer(s) (**Fig. 10B-C'**). *ebony* null mutants develop a darker pigmentation compared to wild type controls (WT, **Fig. 10A**), 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 (**Fig. 10D-D', J-K**), confirming the previous results [Akiyama et al., 2022]. I wondered whether important sequences that maintain WT levels of *ebony* expression reside outside of the deleted region. To test this, I 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 (**Fig. 10E-F', J-K**).

Even though these deletions only had a mild effect in the adult pigmentation, I wondered if they had any effect on *ebony* expression. I analyzed *ebony* mRNA in the abdomen of flies at the eclosion stage using *in situ* hybridization. While

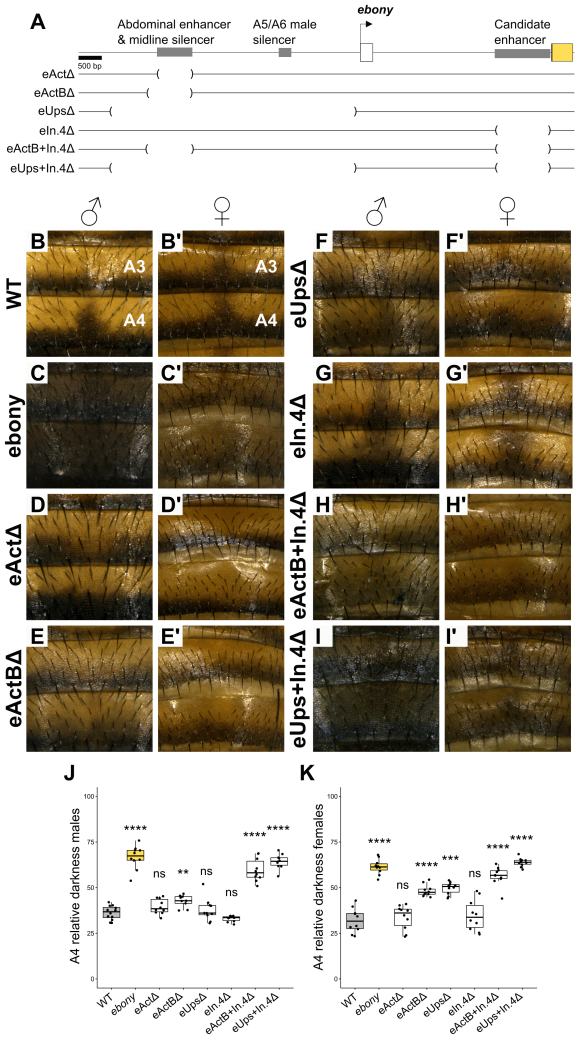

Figure 10: *ebony* abdominal expression is controlled by redundant enhancers. **(A)** Gene map of the *ebony* locus showing the location of the deletions created to identify redundant enhancers. **(B-I')** A3 and A4 pigmentation of males and females of WT, *ebony* null, and deletion lines. **(J-K)** 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 $<$ 0.05, **p $<$ 0.005, ***p $<$ 0.0005, ****p $<$ 0.00005)

all deletion backgrounds showed WT levels of expression, deletions overlapping the *eAct* region resulted in *ebony* de-repression along the dorsal midline (**Fig. 11**). These expression patterns correlate with the adult pigmentation of these lines in which the dorsal midline melanic stripe is erased (**Fig. 10B-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.

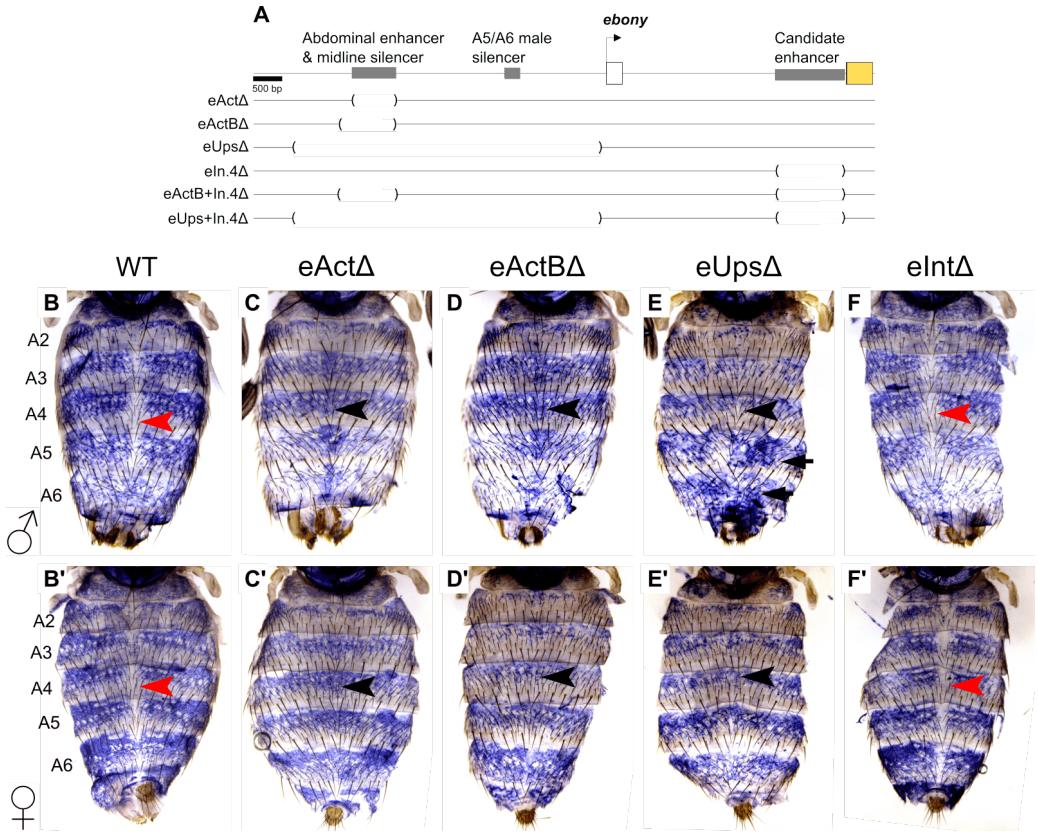

Figure 11: *ebony* abdominal *mRNA* expression correlates with pigmentation phenotypes. **(A)** Gene map of the *ebony* locus showing the location of the deletions created to identify redundant enhancers. **(B-F')** *ebony* abdominal *mRNA* expression measured with *in-situ* hybridization in recently eclosed adults for males and females of WT and deletion lines.

To identify the redundant enhancer(s), I focused on a candidate region located within the first *ebony* intron (*eIN.4*, **Fig. 10A**). 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 in-

tronic stripe silencer *eSS* (see below). I 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 (**Fig. 10G-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 (**Fig. 10H-K**). Thus, *eIN.4* functions as a partially redundant enhancer working together with *eActB* to drive robust *ebony* expression in the abdomen.

Although I focused on the abdominal pigmentation, I noticed that other tissues including the head, thorax, legs, halteres, and wings of *eUps+IN.4*Δ had a darker pigmentation compared to WT (**Fig. 12**). 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 (**Fig. 12**). 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.

2.2.2 *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, I created a deletion targeting this region (**Fig. 13A**). While the A5-A6 pigmentation was not affected in *eMS*Δ (**Fig. 13B-C, F**), I observed higher *ebony* mRNA expression compared to WT as measured by *in situ* hybridization (**Fig. 13D-E**). These experiments confirm that *eMS* is

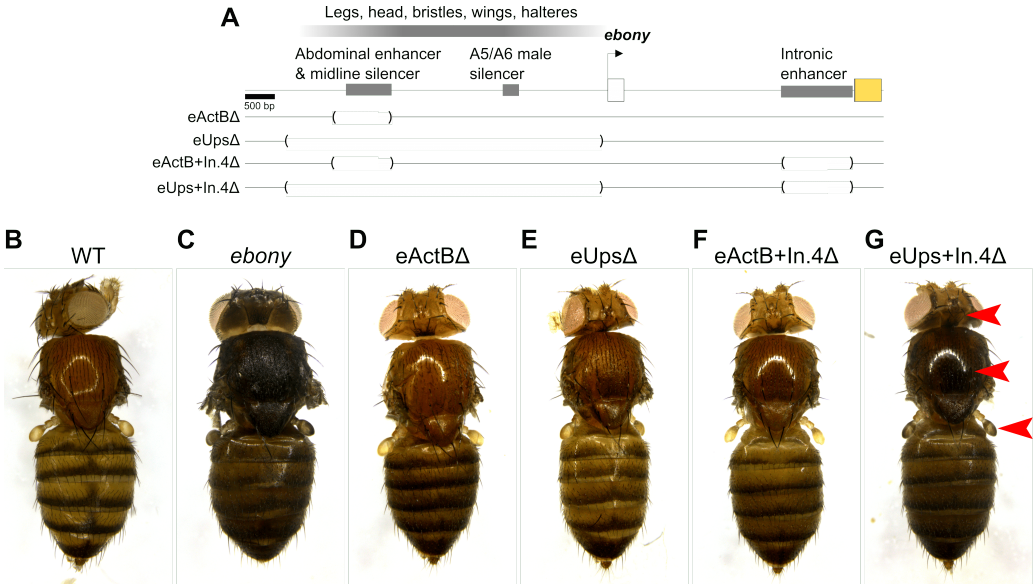

Figure 12: The redundant intronic enhancer is active in multiple adult tissues. **(A)** Gene map of the *ebony* locus showing the location of the deletions created to identify redundant enhancers. Previously identified tissue-specific enhancers are shown on top of the *ebony* upstream region (shaded rectangle). **(B-G)** Pigmentation of different adult tissues in females from WT, *ebony* null, and deletion lines. Red arrows show tissues, other than the abdomen, with darker pigmentation compared to the WT and more similar to *ebony* mutants.

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]. I narrowed

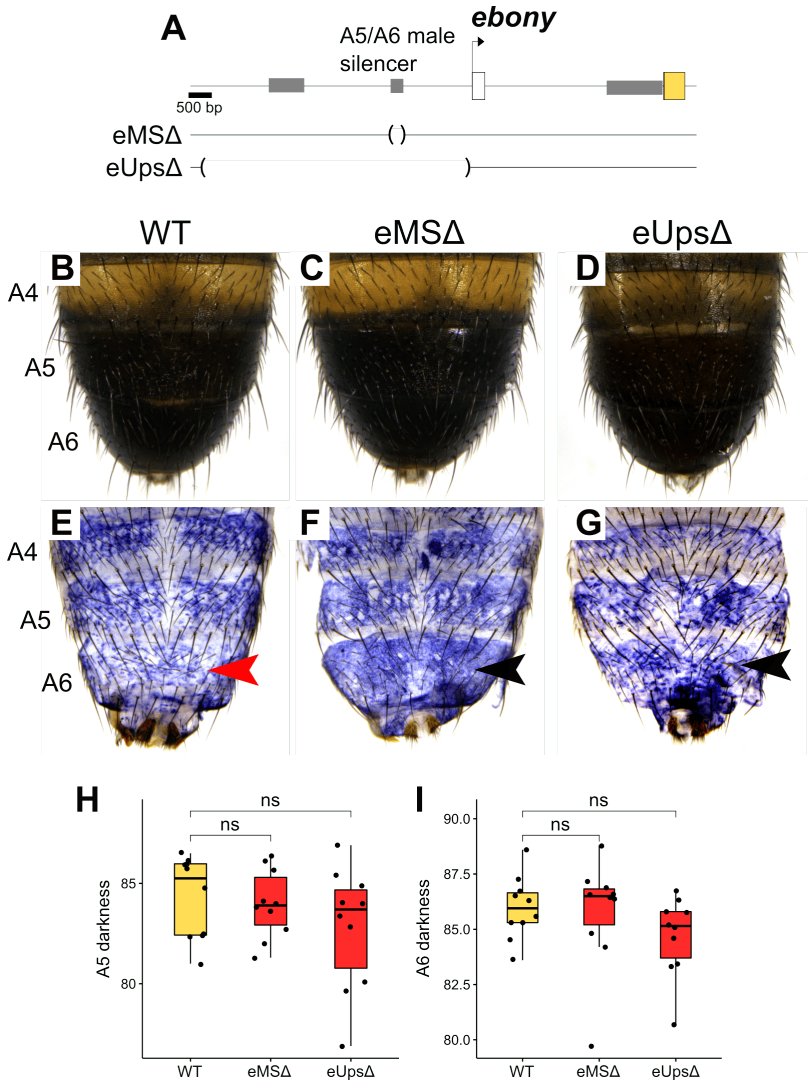

Figure 13: Necessity of the *ebony* A5/A6 male silencer. **(A)** Gene map of the *ebony* locus showing the location of the deletion targeting the A5/A6 male silencer. **(B-D)** A4, A5 and A6 pigmentation of WT, *eMS*Δ, and *eUps*Δ males. **(D-E)** *in-situ* hybridization detecting *ebony mRNA* in A4, A5 and A6 segments of WT, *eMS*Δ, and *eUps*Δ males. Red and black arrowheads indicate low and increased levels of *ebony mRNA*, respectively. **(F)** Comparison of A5 and A6 darkness between WT and *eMS*Δ males. (Student's t test, ns = not significant).

down the exact location of this silencer using nuclear-localized Green Fluorescent Protein (or GFP) reporter constructs 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 (**Fig. 14**). The endogenous deletion of this region resulted in *ebony* de-repression in the stripe area and thinner melanic stripes compared to the WT (**Fig. 15**), 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.

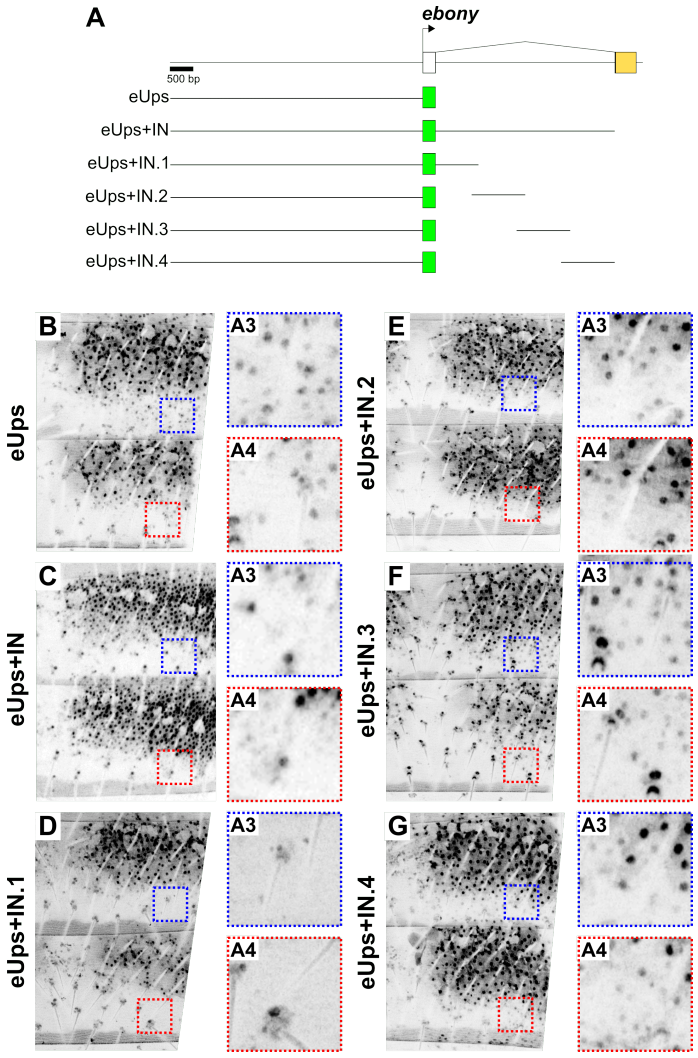

Figure 14: Identification of the stripe silencer within the first *ebony* intron. **(A)** Gene map of the *ebony* locus showing the location of the reporter constructs created to identify the stripe silencer within the first intronic region. **(B-G)** GFP expression pattern of the different transgenic reporters at 24h after eclosion. Blue and red dashed boxes show a magnification of the stripe area in A3 and A4, respectively.

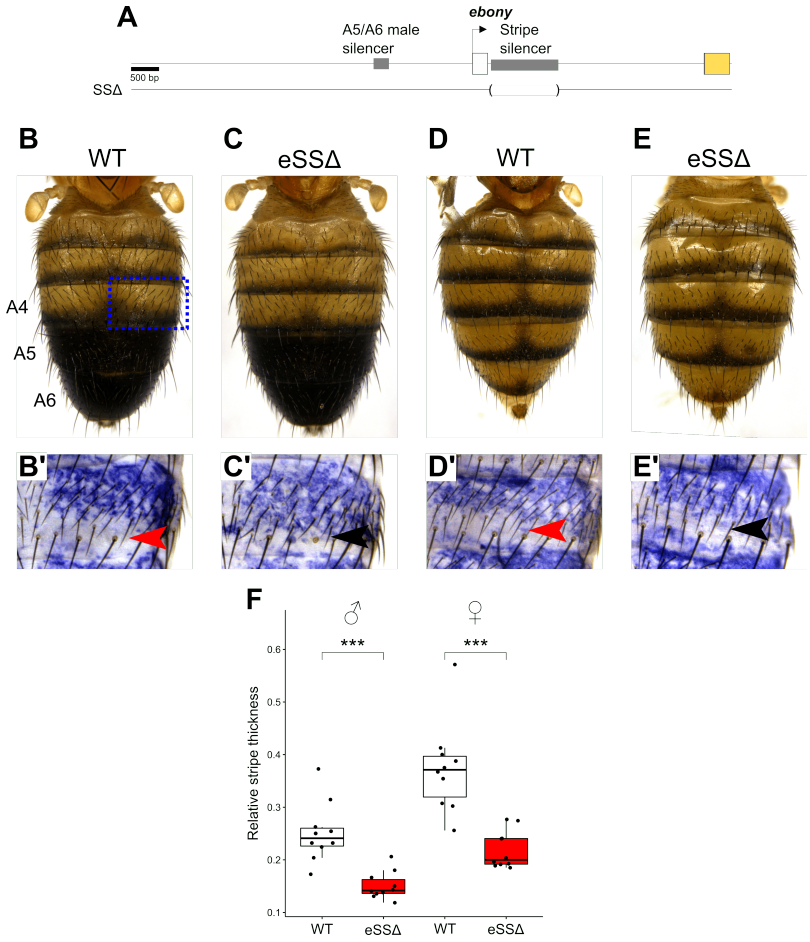

Figure 15: Necessity of the *ebony* stripe silencer. **(A)** Gene map of the *ebony* locus showing the location of the deletion targeting the stripe silencer. **(B-E)** Adult pigmentation of WT and *eSSΔ* males and females. **(B'-E')** *In-situ* hybridization detecting *ebony mRNA* in the A4 segment of WT 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, *** = $p < 0.0005$)

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

To understand how *ebony* expression has evolved, I 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, I selected two species from this group with contrasting abdominal pigmentation, *D. ananassae* (non-melanic) and *D. malerkotliana* (A4, A5 and A6 melanic). I also included *D. pseudoobscura*, a completely melanic species which displays very low levels of *ebony* expression [Hughes et al., 2020] (**Fig. 16A**). I 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*, (**Fig. 16B**). These constructs were tested for GFP activity in the A4-A6 segments of transgenic *D. melanogaster* males 24 hours (h) after eclosion.

I found that the activator region of *D. ananassae* drives reporter expression in all abdominal segments (**Fig. 16C**). Qualitatively, this expression pattern did not change when the full upstream region (**Fig. 16D**) or upstream together with the intronic regions were analyzed (**Fig. 16E**). These results suggest that in *D. ananassae*, *ebony* abdominal expression is controlled by an upstream enhancer (**Fig. 16L**).

For *D. malerkotliana*, I found that the activator and the upstream region drive uniform GFP expression in all abdominal segments (**Fig. 16F-G**). This reporter activity does not recapitulate the endogenous expression of *D. malerkotliana ebony*, which is restricted from the A4, A5, and A6 segments (**Fig. 16B**) [Signor et al., 2016]. However, when the intronic region was included, the expression in A5 and A6

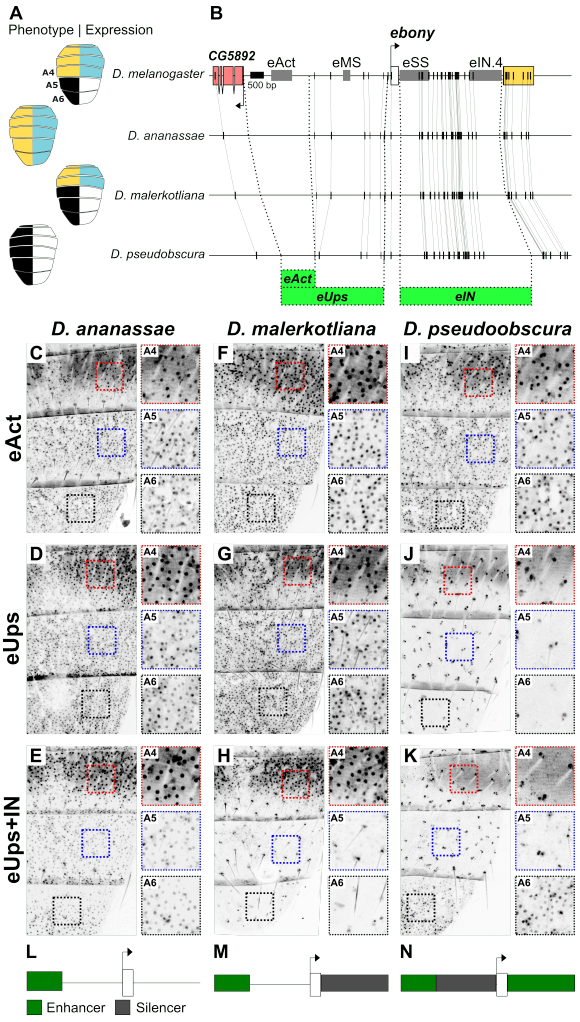

Figure 16: Changes in the location and function of transcriptional silencers among *Drosophila* species. **(A)** Pigmentation (left) and *ebony* expression pattern (right, blue color) of studied *Drosophila* species. **(B)** Sequence alignment showing *D. melanogaster* CREs, conserved regions (vertical lines), and fragments tested for reporter activity (dashed lines). **(C-K)** GFP expression patterns of transgenic reporters in abdominal segments A4-A6. Insets show magnified regions for each segment. **(L-N)** Inferred approximate location of *ebony* abdominal enhancers (green) and silencers (gray).

was silenced (**Fig. 16H**), 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. I noticed that the *D. malerkotliana eUps+IN* reporter also repressed GFP expression in the stripe area (**Fig. 16D, G**). This suggests that this species contains intronic silencer(s) active in both the A5-A6 segments and in the stripe area. I 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 (**Fig. 17**). 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* (**Fig. 17**). These results suggest that in *D. malerkotliana*, *ebony* abdominal expression is controlled by an upstream enhancer and at least two tissue-specific silencers (**Fig. 16M**).

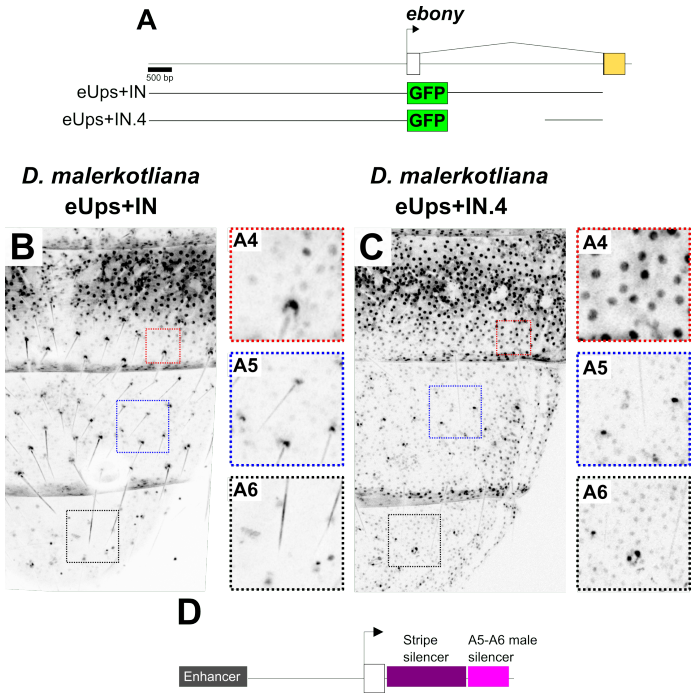

Figure 17: The *ebony* male and stripe silencers of *D. malerkotliana* are located in distinct intronic regions. **(A)** Gene map showing the reporter constructs created to identify the location of the *D. malerkotliana* male silencer within the first *ebony* intron. **(B-C)** GFP expression pattern of *D. malerkotliana* transgenic reporter eUps+IN and eUps+IN4. 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.

For *D. pseudoobscura*, I found that the activator region drives GFP expression in A4-A6 segments in a similar pattern to *D. ananassae* and *D. malerkotliana* (**Fig. 16I**). This was surprising considering how the endogenous expression of *ebony* in *D. pseudoobscura* is almost undetectable [Hughes et al., 2020]. However, when the

full upstream region was analyzed, I found no GFP expression throughout the abdomen (**Fig. 16J**). 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, I observed GFP expression only in A6 albeit at low levels (**Fig. 16K**). I analyzed the reporter expression of the intronic region alone and found it to be A6 specific (**Fig. 18**). 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 (**Fig. 16N**).

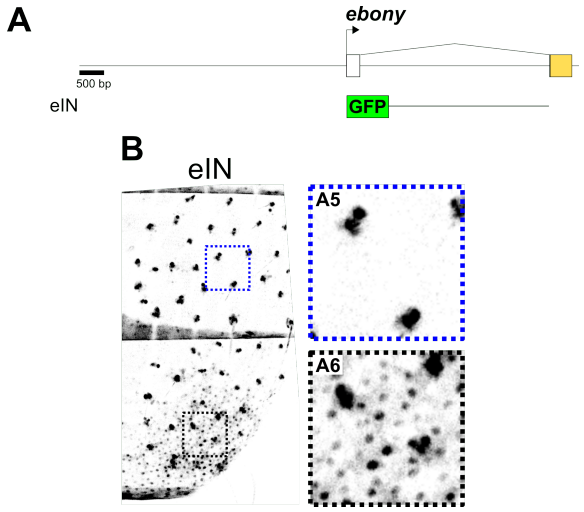

Figure 18: Enhancer activity of the *ebony* intronic region from *D. pseudoobscura*. **(A)** Gene map showing the reporter constructs created for *D. pseudoobscura*. **(B)** GFP expression patterns of *D. pseudoobscura* transgenic reporter eIN.

2.2.4 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* (**Fig. 9B**) is regarded as characteristic of species within the subgenus *Sophophora* [Markow and O'Grady, 2005]. However, I 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* (**Fig. 11**) [Akiyama et al., 2022], I wondered about the evolution of this silencer function. I found that the *eAct* transgenic reporter of the three species studied here drive robust GFP expression along the dorsal midline (**Fig. 19A**), suggesting that none of these species contain a functional midline silencer. To expand our phylogenetic sample, I 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 (**Fig. 19B**). 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.

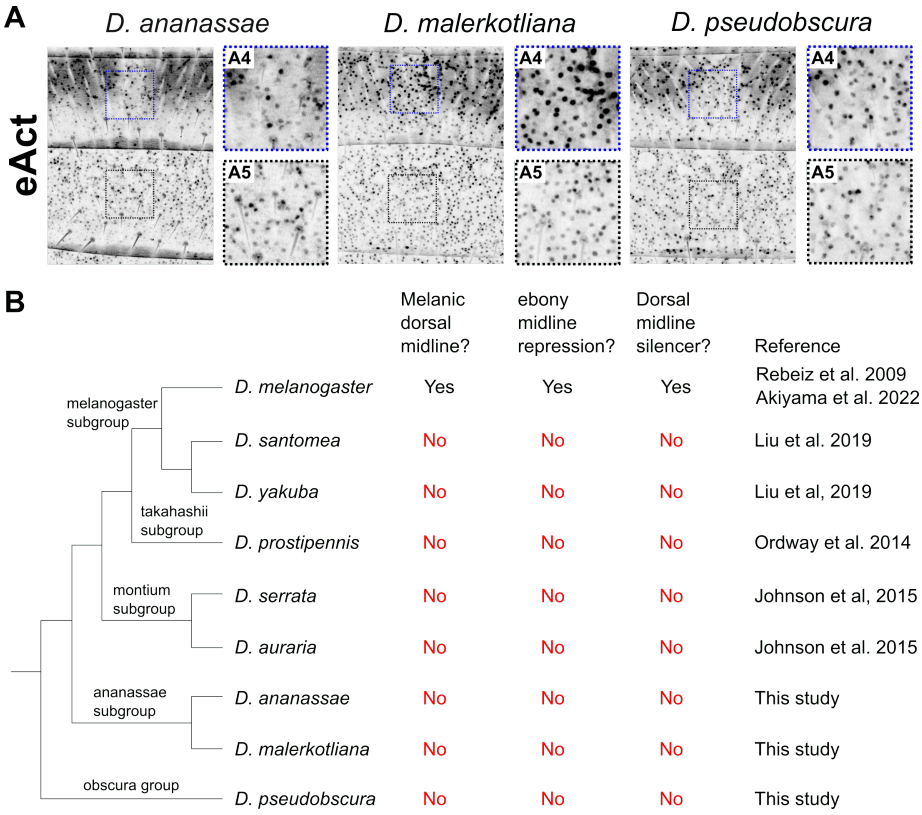

Figure 19: The melanic dorsal midline is novel to *D. melanogaster*. **(A)** GFP expression patterns of the eAct transgenic reporters in the abdominal segments A4-A5. Insets show magnified regions along the midline for A4 (red square) and A5 (blue square). **(B)** Phylogenetic distribution of the melanic dorsal midline in *Drosophila* species for which the expression and regulation of *ebony* in this area has been studied.

2.3 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]. Here I 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, I 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 (**Fig. 20**).

2.3.1 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 (**Fig. 20A**). 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*, I 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

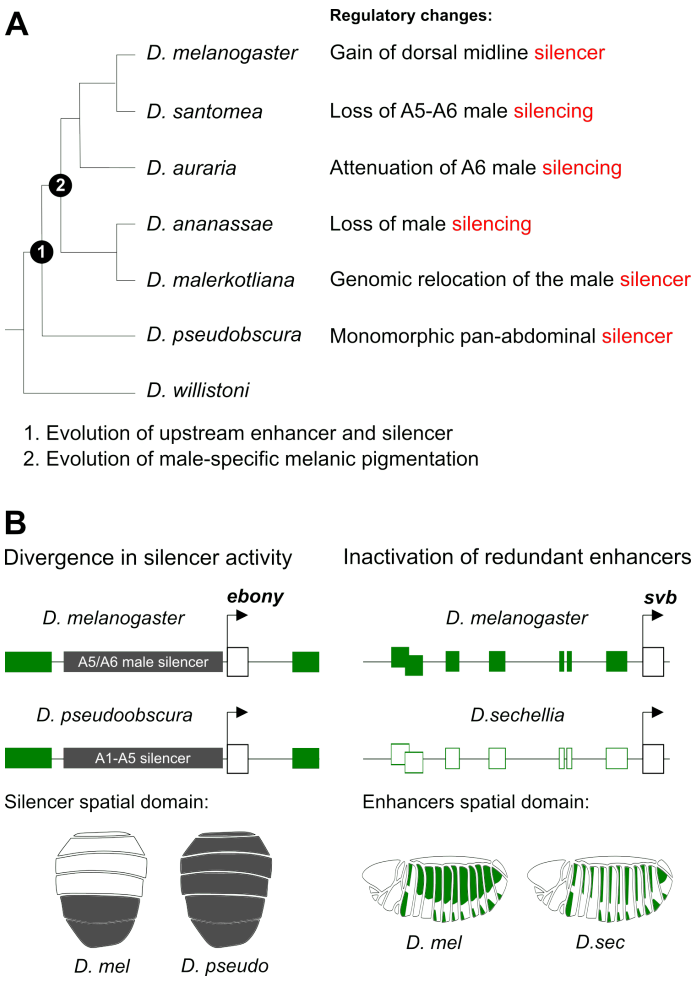

Figure 20: Changes in the function of silencers drive the regulatory evolution of *ebony* abdominal expression. **A)** Summary of *ebony* regulatory changes in *Drosophila* species from this and previous studies, and in relation to the evolution of male-specific melanic pigmentation. **B)** Morphological 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.

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.

2.3.2 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, I 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 (**Fig. 20B**). 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.

2.3.3 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.

3.0 Temporal shifts in Abd-B expression drive the evolution of melanic abdominal pigmentation

3.1 Introduction

During animal development, *Hox* genes control the expression of dozens of genes, dictating the position where unique traits will form along the anterior-posterior body axis [Bender et al., 1983]. While this function is deeply conserved [He et al., 2018, Arnold et al., 2021], changes in *Hox* gene expression often correlate with morphological differences [Carroll, 1995, Averof and Patel, 1997, Stern, 1998]. However, direct evidence showing that *Hox* regulatory evolution has contributed to morphological changes between species remains surprisingly scarce. Moreover, classic mutations affecting *Hox* expression result in major phenotypic changes ("homeotic mutants") [Lewis, 1978] which are unlikely to evolve by natural selection [Akam, 2002]. What kind of regulatory mutations can produce divergent *Hox* expression patterns and, at the same time, reduce deleterious effects?

The melanic abdominal pigmentation of *Drosophila* provides an excellent model to study the molecular basis of *Hox* regulatory evolution. In males from different *Drosophila* species, the *Hox*-gene Abd-B regulates the production of melanin covering the tergites of abdominal segments 5 and 6 (hereafter referred as A5-A6) [Kopp et al., 2000, Liu et al., 2019]. Character reconstruction analysis suggests that A5-A6 melanic pigmentation evolved in the *melanogaster* species group, within which it has been secondarily lost multiple times [Jeong et al., 2006] (**Fig. 21A**). Although the deep conservation of *Hox* expression patterns has led the field to anticipate invariance in insect *Hox* gene expression patterns, recent work has challenged this view

[Stern, 1998, Tanaka et al., 2011, Liu et al., 2019, Tian et al., 2019]. In *D. santomea*, the loss of A5-A6 melanic pigmentation involved regulatory mutations disrupting Abd-B expression during late pupal stages [Liu et al., 2019]. Likewise, variation in mimetic color among bumble bees correlates with quantitative temporal differences in Abd-B expression [Tian et al., 2019]. These studies suggest that expression differences occurring during late developmental stages might be a mechanism to reduce the possible deleterious effects associated with highly pleiotropic genes. However, examples of how such differences are generated at the molecular level are currently lacking.

Here I examined the extent to which variation in Abd-B expression has contributed to the evolution of melanic pigmentation in *Drosophila*. Surprisingly, I found that Abd-B A5-A6 expression during late pupal development evolved quite recently in the *melanogaster* species subgroup where it correlates with the presence of melanic pigmentation. Using CRISPR-*Cas9* mediated endogenous deletions and replacements, I identified two CREs necessary for Abd-B A5 pupal expression and the formation of melanin in *D. melanogaster* and which function is conserved even in species lacking Abd-B A5 pupal expression. Thus, this substantial change in expression seems to have evolved in the face of conserved CREs, suggesting the existence of unknown interactions between these regions and silencers, and/or changes in upstream factors. Altogether, I propose that temporal differences in *Hox*-gene expression might represent a general mechanism to modify a specific aspect of the segment morphology without resulting in homeotic mutants.

3.2 Results

3.2.1 Abd-B A5-A6 pupal expression is novel to the *melanogaster* species subgroup

In *D. melanogaster*, the *Hox* gene Abd-B is required for specifying the identities of posterior abdominal segments (A5-A9) [Celniker et al., 1989]. Approximately 60 hours after pupal formation (hAPF, hereafter mid-pupal development), Abd-B regulates the formation of melanic pigmentation covering the male's A5 and A6 (**Fig. 21B-B'**) [Kopp et al., 2000]. Within the *melanogaster* subgroup, most species develop abdominal melanic pigmentation (**Fig. 22A**). A striking exception is *D. santomea* which has secondarily lost A5-A6 melanic pigmentation. This phenotype is associated with a loss of Abd-B A5 expression during mid-pupal development (**Fig. 21D-D'**), and the gain of expression of the melanic-repressor pdm3 in A6 [Liu et al., 2019].

Beyond *D. santomea*, it is unclear whether differences in Abd-B A5-A6 expression have evolved in other species. A former member of the Rebeiz Lab, Dr. Yang Liu used immunofluorescence to assess A5-A6 mid-pupal expression in all the other species from the *melanogaster* subgroup. A5-A6 expression was observed in all the species tested except for *D. orena* A5, which correlates with its non-melanic A5 pigmentation (**Fig. 22B-G**). Thus, species from the *melanogaster* subgroup exhibit a perfect correlation between Abd-B A5-A6 mid-pupal expression and the formation of melanic pigmentation. Next, I tested whether a similar correlation is found in more distantly related species. A5-A6 melanic pigmentation seems to have evolved in the *melanogaster* species group after splitting from the non-melanic *willistoni* lineage (**Fig. 21A**) [Jeong et al., 2006]. Thus, I selected *D. willistoni* as the out-

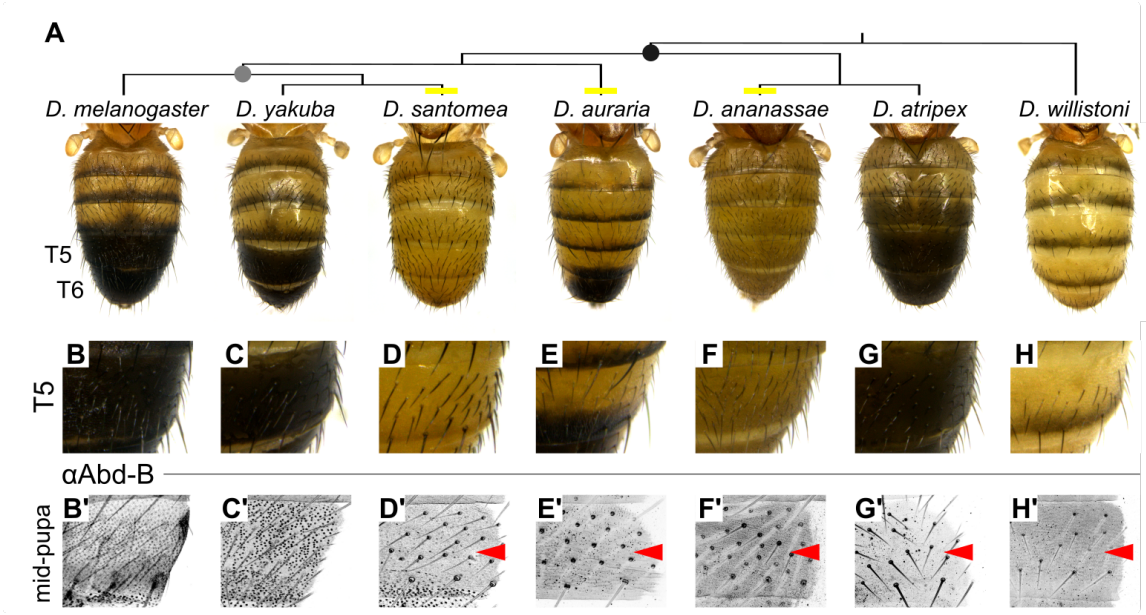

Figure 21: Abd-B A5 pupal expression evolved within the *melanogaster* species subgroup. **(A)** Abdominal pigmentation of males from different *Drosophila* species. The black and gray circles indicate the melanogaster species group and subgroup, respectively. Inferred secondary losses of A5 and A5-A6 melanic pigmentation are marked with a yellow bar. **(B-H)** A5 pigmentation. **(B'-H')** A5 Abd-B expression during mid-pupal development. Red arrowheads indicate lack of Abd-B expression.

group species, *D. auraria* and *D. ananassae* as species with secondary losses, and *D. atripex* as a melanic species (**Fig. 21D-F**). To analyze Abd-B expression in these species, I developed a new antibody and confirmed its cross-reactivity in embryos (**Fig. 23**). I did not observe Abd-B A5 mid-pupal expression in any of the species tested (**Fig. 21E'H'**). While these results correlate with the non-melanic A5 pigmentation of *D. auraria*, *D. ananassae*, and *D. willistoni*, the lack of expression in *D. atripex* (melanic) is unexpected. These results suggest that Abd-B A5-A6 mid-pupal expression evolved specifically within the *melanogaster* species subgroup, after the initial evolution of melanic pigmentation; and that species like *D. atripex* regulate the development of abdominal melanic pigmentation in a *Hox*-independent or more indirect manner.

3.2.2 Two CREs regulate Abd-B A5 pupal expression in D. melanogaster

To understand how Abd-B A5 pupal expression evolved, I first investigated the regulation of this expression pattern in *D. melanogaster*. Abd-B expression in the different tissues composing the A5 segment is controlled by a large *cis*-regulatory module known as *infrabdominal-5* (*iab-5*)[Casares and Sánchez-Herrero, 1995]. The activation of Abd-B A5 expression during early embryogenesis relies on the initiator element IAB5 [Busturia and Bienz, 1993]. IAB5 is necessary, but not sufficient for Abd-B expression during late developmental stages, which is regulated by tissue-specific *iab-5 cis*-regulatory elements (CREs) [Mihaly et al., 2006, Maeda and Karch, 2006, Postika et al., 2021]. Interestingly, the function of IAB5 seems to be highly conserved even among distantly related *Drosophila* species [Ho et al., 2009]. Thus, differences in Abd-B A5 pupal expression could have evolved by targeting pupal-specific CREs that are not active during earlier developmental stages.

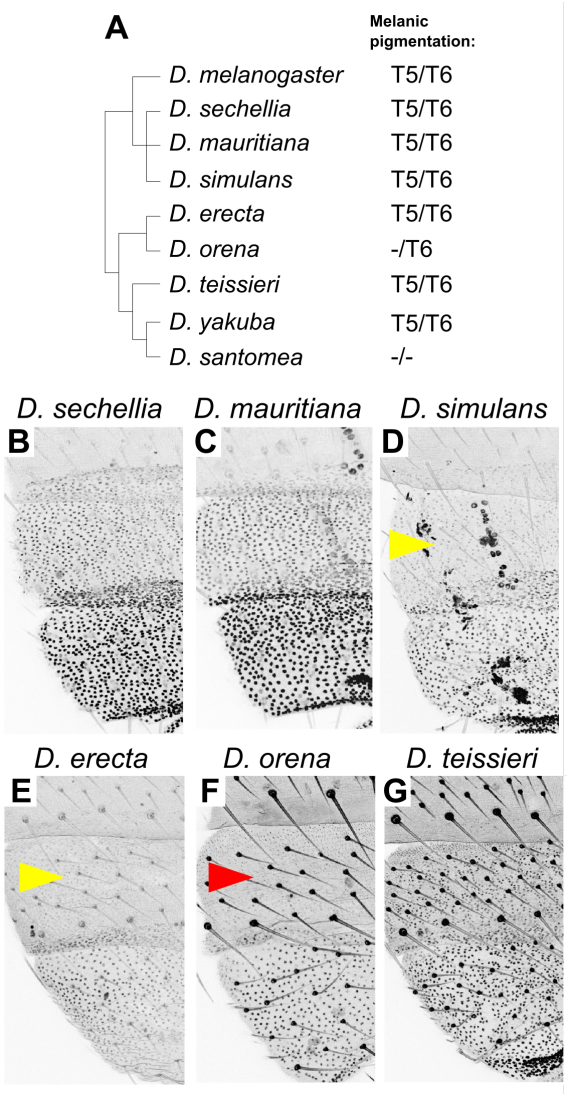

Figure 22: Abd-B pupal expression in species from the melanogaster subgroup. **(A)** Phylogeny of the *melanogaster* species subgroup showing the presence/absence of melanic pigmentation in A5 and A6. **(B-G)** Abd-B expression during mid-pupal development in A5 and A6 for each species. Red and yellow arrows indicate lack and low expression in A5, respectively.

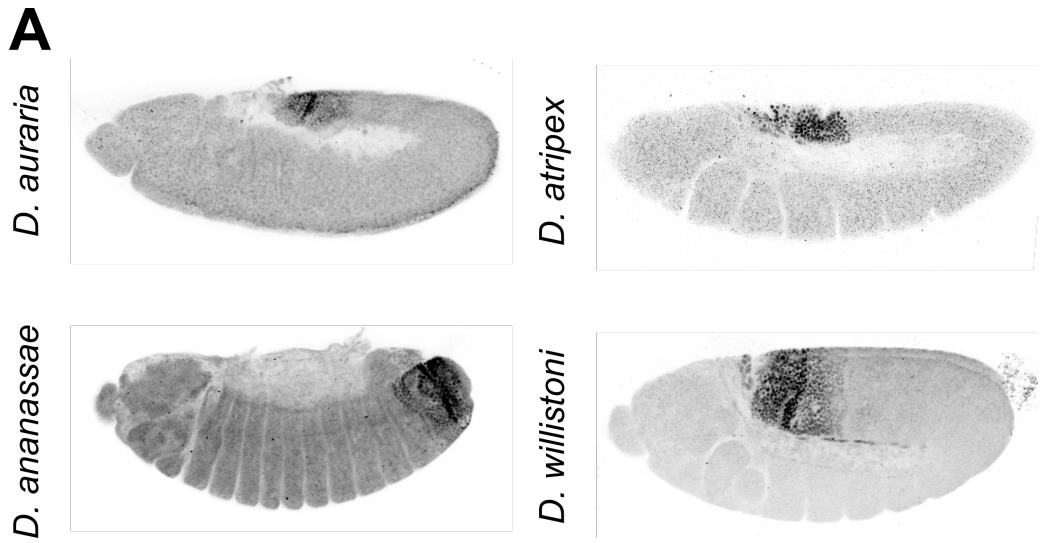

Figure 23: Validation of the Abd-B polyclonal antibody in different *Drosophila* species. **(A)** Cross-reactivity of the Abd-B polyclonal antibody in embryos from different *Drosophila* species.

A recent study identified multiple *iab-5* CREs regulating Abd-B expression and the formation of melanic pigmentation [Postika et al., 2021]. However, the necessity and temporal activity of these CREs was not directly assessed. Likewise, CREs active during late developmental stages require the early activity of IAB5 [Casares and Sánchez-Herrero, 1995], and thus, to understand the functioning of these CREs it is necessary to study them in their endogenous context. To characterize *iab-5* A5 pupal CREs I created four overlapping deletions via CRISPR-*Cas9* mediated homologous recombination spanning the entire *iab-5* region and excluding IAB5 (**Fig. 24A**). Two deletions, *iab-5* Δ1 and *iab-5* Δ3, decreased the formation of A5 melanic pigmentation compared to the wild-type (**Fig. 24B-F'**). While *iab-5* Δ1 completely eliminates the melanic pigmentation (**Fig. 24C'**), *iab-5* Δ3 results in variable patches of non-melanic cuticle (**Fig. 24E'**). Quantitative comparison of the A5 darkness in wild-type and deletion strains confirmed the larger effect size of *iab-5* Δ1 (**Fig. 24G**). Thus, *iab-5*.3 is necessary for A5 pupal expression although it seems to require the function of *iab-5*.1.

Next, I analyzed Abd-B expression across different pupal stages in each deletion line. I found wild-type expression at all time points for the *iab-5* Δ2 and *iab-5* Δ4 deletions which develop a wild-type melanic pigmentation (**Fig. 24J, L** and **Fig. 25**). As expected from their phenotypes, *iab-5* Δ1 completely lacks A5 expression in all time points tested, while for *iab-5* Δ3, I observed variable patches of A5 cells lacking expression (**Fig. 24I** and **Fig. 25**). Interestingly, during the earliest time point tested, Abd-B A5 expression in *iab-5* Δ3 appears wild-type (**Fig. 25E**). Unlike previously reported, none of the *iab-5* deletions had any effect in Abd-B A6 expression (**Fig. 25L-P**) [Postika et al., 2021], highlighting the segment-specific function of these CREs. These results suggests that, in *D. melanogaster*, two segment-specific CREs (*iab-5*.1 and *iab-5*.3) with partially overlapping temporal activities are neces-

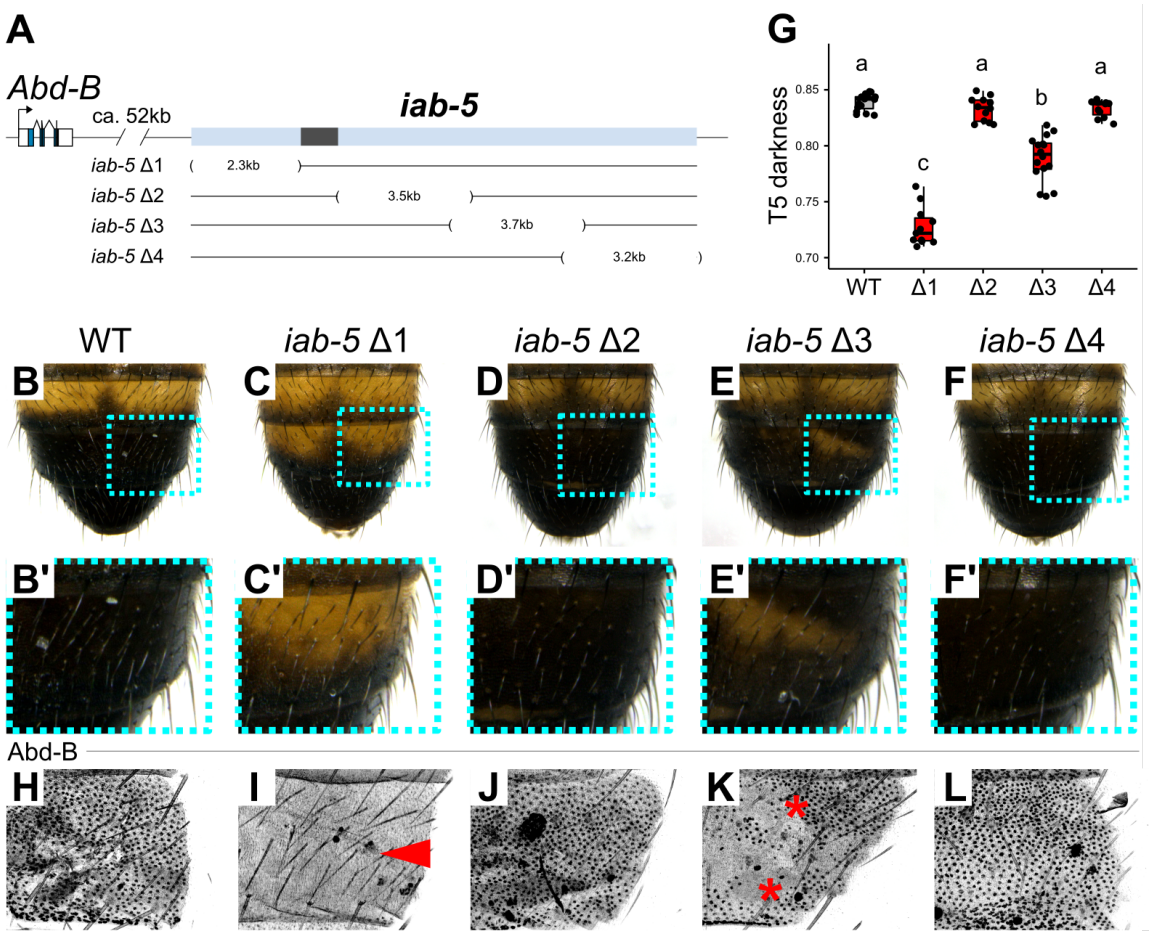

Figure 24: Two *iab-5* CREs are necessary for Abd-B A5 pupal expression and melanic formation in *D. melanogaster*. **(A)** Map of the Abd-B gene and the *iab-5* regulatory domain showing the location and size of the deletions. The IAB5 initiator element (gray bar) was not deleted. **(B-F)** T4-A6 pigmentation of each strain. **(B'-F')** A5 pigmentation of each strain. **(G)** Quantification of A5 darkness. **(H-L)** A5 Abd-B expression at 74 hAPF in each strain. Significant differences in A5 darkness were tested with one-way ANOVA and Tukey's test.

sary for Abd-B A5 pupal expression and that this window of expression is crucial for the formation of melanic pigmentation.

3.2.3 *iab-5*.1 regulates Abd-B expression the pupal A5 segment and the genital disc

While all *iab-5* deletions are homozygous viable, *iab-5* Δ1 produces sterile homozygous flies. Inspection of the adult male genitalia revealed a severe rotation defect (**Fig. 26B-F**). In *D. melanogaster*, Abd-B expression in the genital disc (GD) is required for the rotation of the male genitalia and the establishment of the left/right asymmetry [Coutelis et al., 2008, 2013]. I analyzed Abd-B GD expression in *iab-5* Δ1 and found a severe miss regulation compared to wild-type (**Fig. 26H-I**). To test whether the A5 pupal and GD functions of *iab-5*.1 are controlled by the same or distinct CREs, I created two deletions within *iab-5*.1 (*iab-5* Δ1A and *iab-5* Δ1B) using recombinase mediated cassette exchange (RMCE, **Fig. 27A-C**) [Voutev and Mann, 2018]. As a positive control I reinserted the deleted *iab-5*.1 region into *iab-5* Δ1 via RMCE, which resulted in wild-type A5 pigmentation and normal genital rotation (**Fig. 27G-G'**) . As a negative control, I performed RMCE using an empty donor vector and obtained flies with non-melanic A5 pigmentation and male genitalia with rotation defects (**Fig. 27H-H'**). Both *iab-5* Δ1A and *iab-5* Δ1B produced adults with wild-type male genitalia (**Fig. 27I'-J'**) and A5 pigmentation significantly lighter than wild-type (**Fig. 27D, I-J**). However, compared to *iab-5* Δ1A, *iab-5* Δ1B had a stronger A5 pigmentation phenotype (**Fig. 27D**) . This suggests that most of the A5 regulatory information is contained in the 0.8 kb deleted in *iab-5* Δ1B. Based on these results, I conclude that expression of Abd-B in pupal A5 and the GD is controlled by two *iab-5* CREs located in close proximity

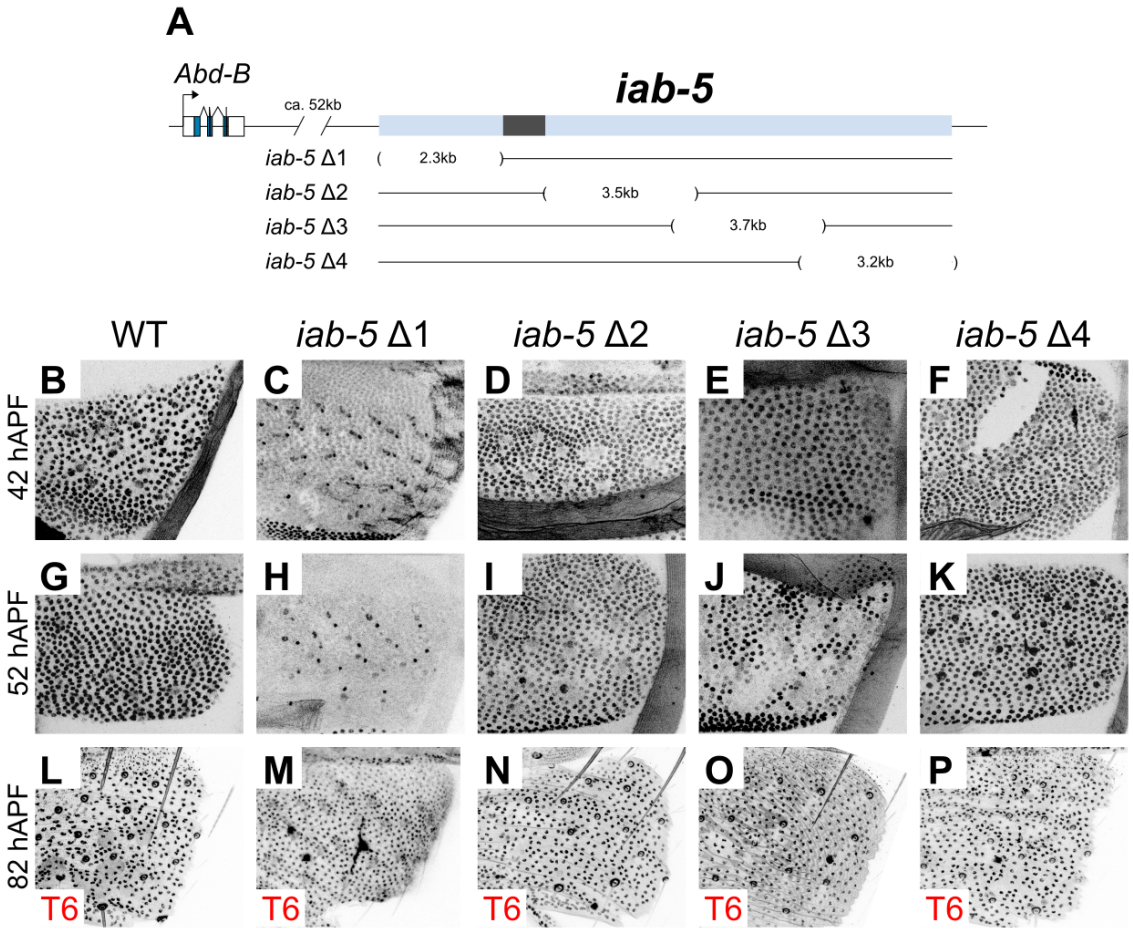

Figure 25: Abd-B A5 expression during pupal development in *iab-5* deletion lines. **(A)** Map of the Abd-B gene and the *iab-5* regulatory domain showing the location and size of the deletions. **(B-F)** A5 Abd-B expression at 42 hAPF in each strain. **(G-K)** A5 Abd-B expression at 52 hAPF in each strain. **(L-P)** A6 Abd-B expression at 82 hAPF in each strain.

(**Fig. 27K**).

3.2.4 The A5 function of *iab-5.1* and *iab-5.3* predates the evolution of Abd-B A5 pupal expression

I hypothesized that the function of *iab-5.1* and *iab-5.3* evolved within the *melanogaster* species subgroup, where it contributed to the gain of Abd-B A5 pupal expression. To test this, I analyzed the functional conservation of these CREs among species within and outside the *melanogaster* subgroup. I replaced *iab-5.1* and *iab-5.3* in *D. melanogaster* with the orthologous sequences from different *Drosophila* species using RMCE (**Fig. 28A**) [Voutev and Mann, 2018]. As a readout of the CRE regulatory activity, I compared the A5 darkness of wild-type, deletion, and replacements strains. While I expected functionally conserved alleles to produce replacement lines with wild-type A5 pigmentation, lack of functional conservation should result in A5 pigmentation defects reminiscent of the deletion.

Contrary to my expectations, I found that *iab-5*.1 is not functionally conserved in *D. yakuba* (**Fig. 28B**), a melanic species with Abd-B pupal expression that belongs to the *melanogaster* subgroup. A similar result was found for the two outgroup species, *D. pseudoobscura* and *D. willistoni*, as well as the non-melanic *D. auraria*. Interestingly, replacements with the *D. ananassae* and *D. atripex* alleles resulted in A5 wild-type pigmentation (**Fig. 28B**), suggesting a high degree of functional conservation among these species. The simplest interpretation of these results is that the A5 pupal function of *iab-5*.1 evolved in the *melanogaster* species group and it has been lost at least twice, in *D. yakuba/D. santomea*, and in *D. auraria*. Since the allele replacements were performed in the context of the entire *iab-5*.1, I also analyzed the conservation of the *iab-5*.1 genital disc function. All the replacement

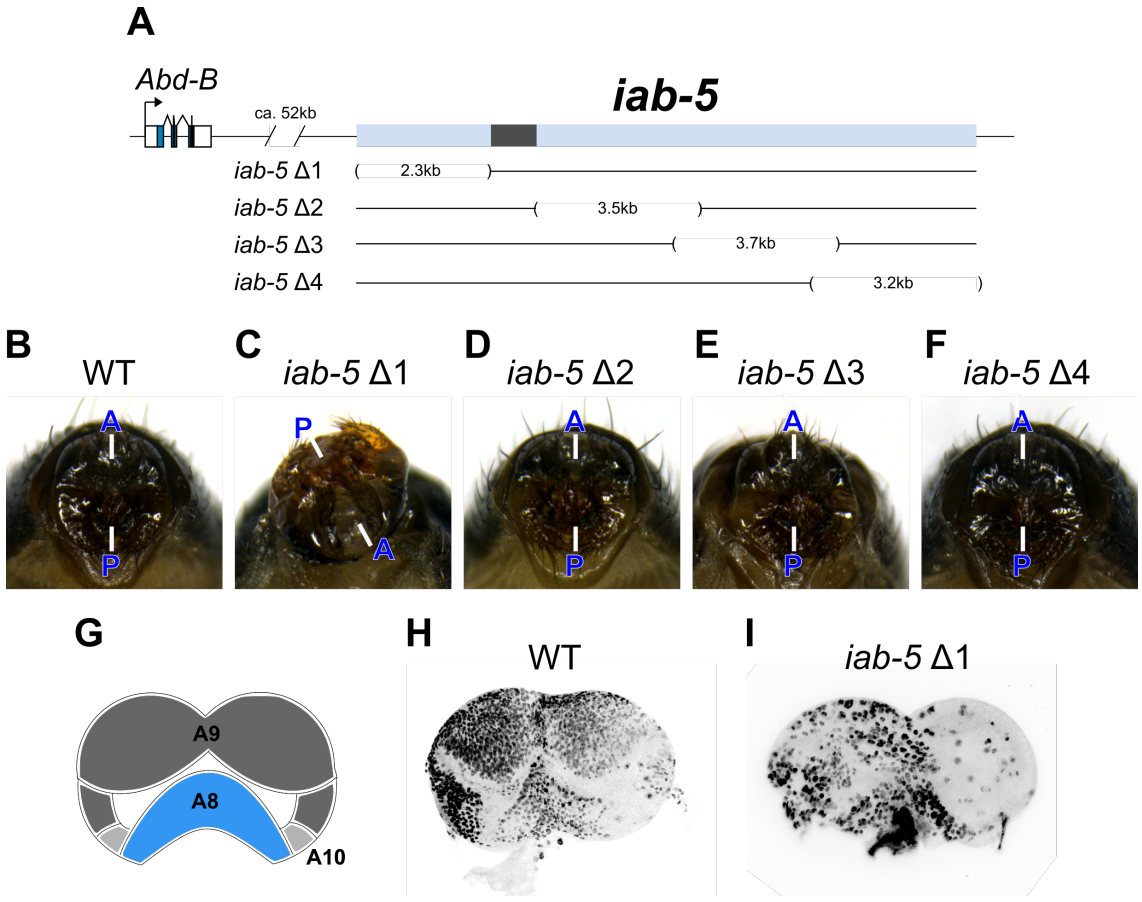

Figure 26: The *iab5.1* CRE is necessary for male genitalia rotation and Abd-B expression in the genital disc. **(A)** Map of the Abd-B gene and the *iab-5* regulatory domain showing the location and size of the *iab-5* deletions. **(B-F)** Male genitalia of each strain. The axis marked by anus (A) and penis (P) is depicted. **(G)** Cartoon showing the location of A8, A9 and A10 segments in the genital disc. **(H-I)** Abd-B expression in WT (H) and *iab-5* Δ1 (I) genital discs.

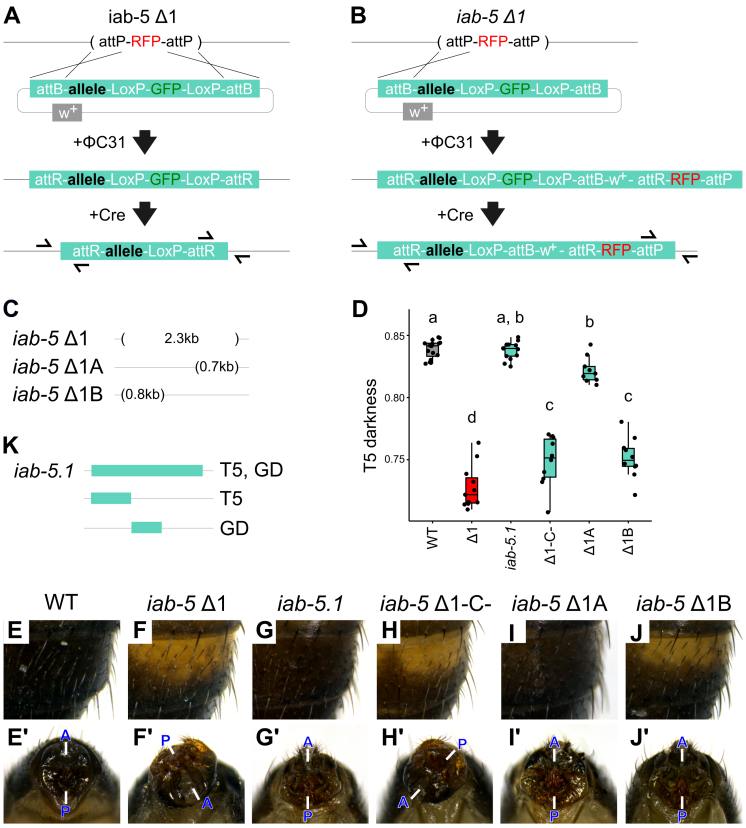

Figure 27: The *iab-5.1* region contains two CREs necessary for A5 and gential disc Abd-B expression. **(A-B)** Representation of the RMCE approache used in the *iab-5* Δ1 locus. Full recombination events **(A)** and plasmid integration events **(B)**. **(C)** Location of *iab-5* Δ1A and *iab-5* Δ1B with respect to *iab-5* Δ1. **(D)** A5 darkness of each strain including the positive control *iab-5.1*, and the negative control *iab-5* Δ1C- (same letters indicate lines which mean values are not significantly different from each other). **(E-J)** A5 phenotypes of each strain. **(E'-J')** Male genitalia of each strain. The axis marked by anus (A) and penis (P) is depicted. **(K)** Inferred location of the A5 and genital disc (GD) CREs. Significant differences in A5 darkness were tested with one-way ANOVA and Tukey's test.

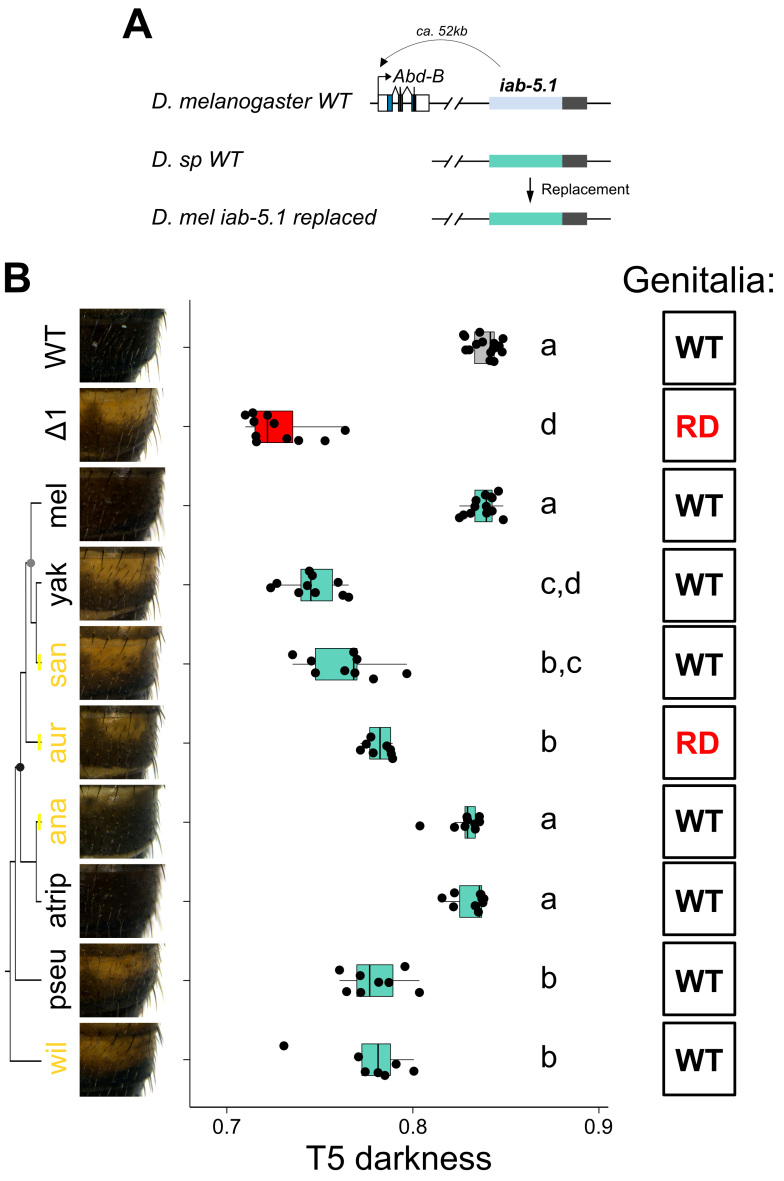

Figure 28: Evolution of *iab5.1* A5 and genital disc functions. **(A)** The *D. melanogaster iab-5.1* CRE was replaced with the orthologous region from different *Drosophila* species. **(B)** A5 pigmentation, quantification, and male genitalia phenotype (WT = wild type, RD = rotation defect) of each strain. Significant differences in A5 darkness were tested with one-way ANOVA and Tukey's test.

lines, except for the *D. auraria* allele, produced adults with wild-type male genitalia (**Fig. 29B** and **Fig. 28B**). Thus, the GD function of *iab-5*.1, seems to have evolved in the common ancestor of the *willistoni-pseudoobscura-melanogaster* groups, prior to the pupal A5 function.

Unlike the high functional divergence of *iab-5*.1 A5, I found that *iab-5*.3 is functionally conserved in all the species tested (**Fig. 30**). Hence, the function of *iab-5*.3 seems to have evolved, at least, in the common ancestor of the *melanogaster-obscura-willistoni* groups. All together, these results suggest that the A5 pupal function of *iab-5*.1 and *iab-5*.3 predates the evolution of Abd-B A5 pupal expression. Likewise, they reveal contrasting patterns of functional conservation in which only the A5 pupal function of *iab-5*.1 seems to have been modified during evolution.

3.3 Discussion

Hox genes perform many functions during development, and mutations affecting their expression often result in major phenotypic changes [Lewis, 1978, Morata and Lawrence, 1977, Gyurkovics et al., 1990]. Likely because of this, the embryonic expression of these genes is highly conserved across insects and other arthropods [Hughes and Kaufman, 2002]. Here I showed that the expression of the *Hox*-gene Abd-B in A5 during late pupal development is a derived state that evolved in the *melanogaster* species subgroup. This expression pattern is regulated by two semi redundant, segment-specific, and temporally restricted CREs (*iab-5*.1 and *iab-5*.3). Unexpectedly, the function of these CREs predates the evolution of Abd-B late pupal expression. I suggest that changes in the function of temporally restricted CREs might represent a mechanism to modify *Hox*-gene expression with minimal

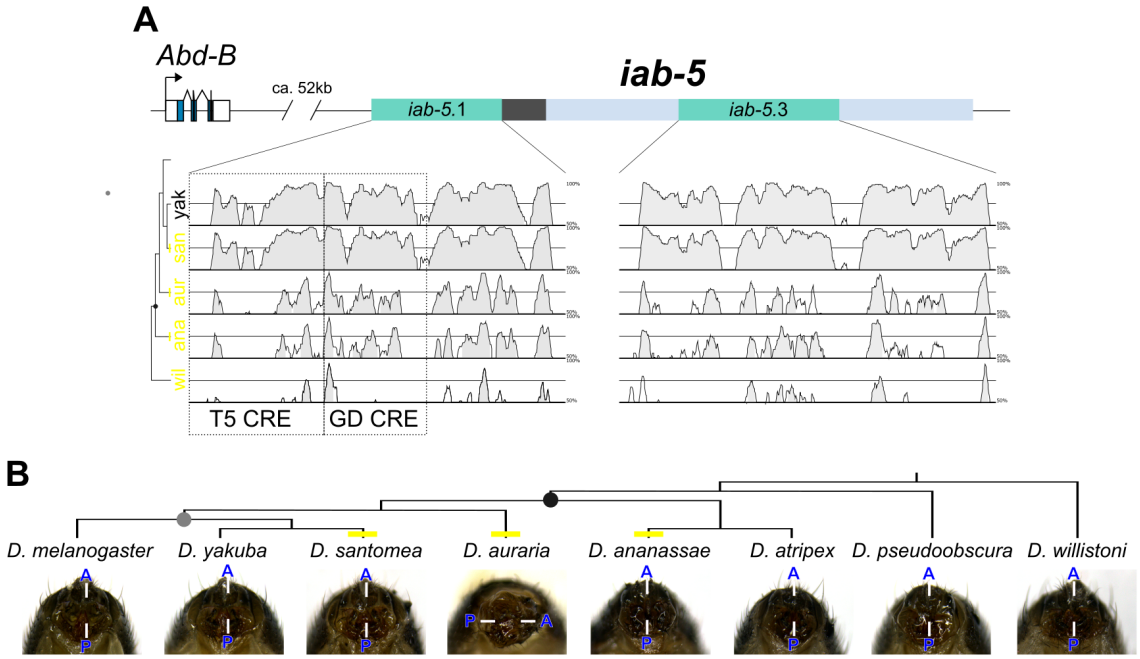

Figure 29: The genital disc function of *iab5.1* CRE evolved at least in the common ancestor of the *melanogaster-obscura-willistoni* species groups. **(A)** VISTA alignment showing the sequence conservation of *iab-5.1* and *iab-5.3* CREs. Dashed boxes show the inferred location of the A5 and genital disc (GD) CREs within *iab-5.1*. **(B)** Genitalia phenotypes of *D. melanogaster* replacement lines. The axis marked by anus (A) and penis (P) is depicted.

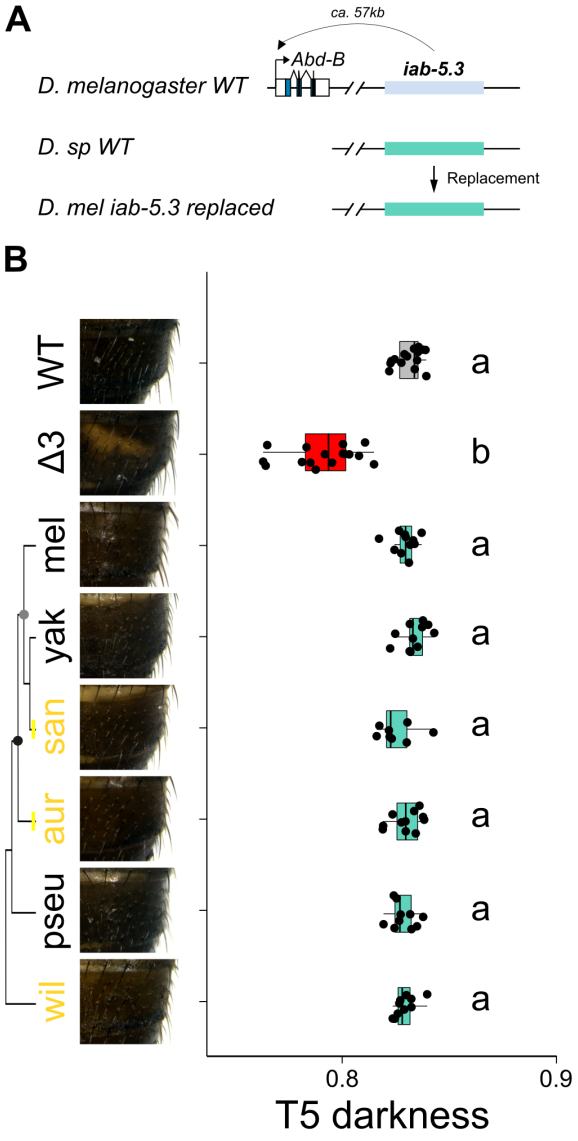

Figure 30: Evolution of *iab-5.3* CRE. **(A)** Map showing the location *D. melanogaster iab-5.3* CRE which was replaced with the orthologous region from different *Drosophila* species. **(B)** A5 pigmentation and quantification of each strain (same letters indicate lines which mean values are not significantly different from each other). Significant differences in A5 darkness were tested with one-way ANOVA and Tukey's test.

pleiotropic effects. Hence, an important aspect of *Hox*-driven morphological evolution could be the modification of traits that are patterned during late development [Tian et al., 2019].

A major question in evolutionary developmental biology is how top tier regulators such as *Hox*-genes acquire novel target genes to fuel morphological variation upon which natural selection can act [Carroll, 1995]. While A5-A6 melanic pigmentation is the most likely ancestral state in the *melanogaster* species group [Jeong et al., 2006], the role of Abd-B as a key regulator of this trait [Celniker et al., 1989, Kopp et al., 2000] is exclusive to the *melanogaster* species subgroup. Hence, the melanic pigmentation of species like *D. auraria* (A6) and *D. atripex* (T4-A6) must be regulated by a different set of factors. This suggest that top tier regulators can undergo extensive turnover even at short evolutionary scales while maintaining similar phenotypic outcomes.

I found that the A5 function of *iab-5*.1 is conserved even in lineages lacking Abd-B A5 pupal expression. The evolution of this novel expression pattern, thus, may have involved changes outside *iab-5*.1 that modified its function in a temporally restricted manner. I suggest two possible scenarios: gain of upstream regulators, and/or loss of repression (**Fig. 31**). In both cases, the A5 function is hypothesized to have evolved from an ancestral and different function. Interestingly, in *D. melanogaster*, the *iab-5*.1 A5 CRE is located next to a highly conserved CRE active in the genital disc. Whether these two functions are separated in species like *D. annanasae* remains to be tested. However, it is tantalizing to consider that the novel A5 function might have evolved by exploiting the activity of an ancestral CRE [Rebeiz et al., 2011].

The possibility that Abd-B pupal expression might have evolved via de-repression is intriguing considering that spatial repression of *Hox*-genes is crucial for their segment-specific expression [Beuchle et al., 2001, Kassis et al., 2017, Gentile and

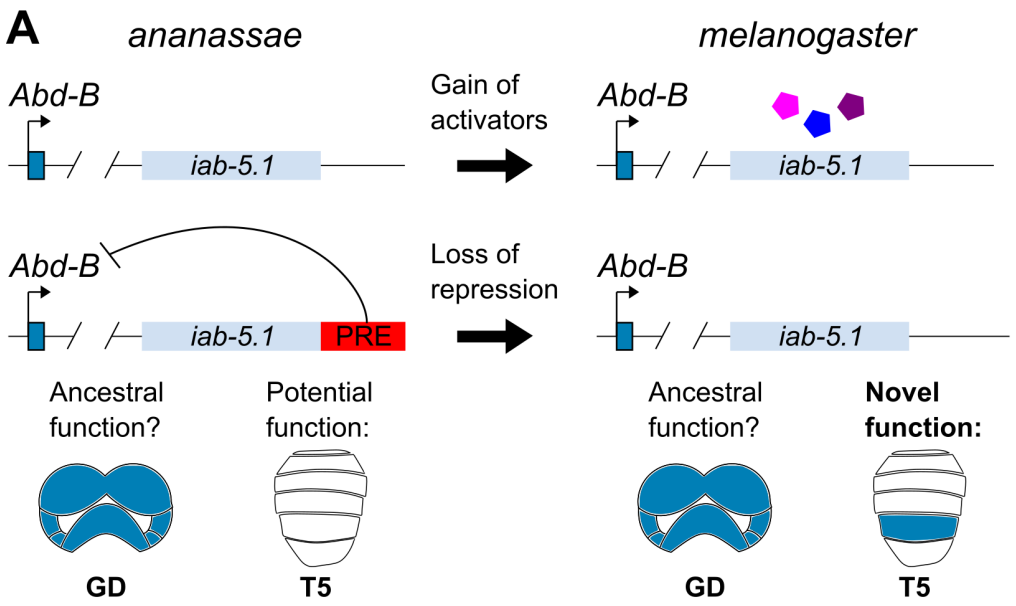

Figure 31: Possible scenarios for the evolutionary gain of Abd-B A5 pupal expression and the regulation of melanic pigmentation. **(A)** The genital disc and pupal A5 functions of *iab-5.1* are conserved between the *ananassae* and *melanogaster* lineages. However, the *ananassae* lineage lacks Abd-B A5 expression during pupal development. Two scenarios could explain the evolutionary gain of Abd-B A5 expression in the *melanogaster* lineage: i) gain of upstream activators (top); ii) loss of repressive sequences (for instance, Polycomb response elements, PREs) that in *ananassae* might prevent *iab-5.1* from activating Abd-B in the pupal A5.

Kmita, 2020] . Proteins belonging to the Polycomb repressive complexes (PRCs) bind to CREs known as Polycomb response elements (PREs) thereby silencing *Hox* expression in certain spatial domains [Strutt et al., 1997, Orlando et al., 1998]. Within *iab-5*, a PRE localized in the boundary element MCP is required for preventing expression of Abd-B in A4 [Busturia et al., 2001]. Little is known, however, about the role of PRC mediated silencing in the temporal dynamics of *Hox*-gene expression. Zhang et al. [2017] found that PRC silencing of Abd-B is necessary for maintaining the cell identity of adult *Drosophila* testis. Likewise, temporal shifts in Abd-B expression between bumble bee species [Tian et al., 2019] and developmental stages in artemia [McCarthy-Taylor et al., 2022] have been described, although their regulatory basis is unknown. Differential gene silencing has been shown to drive expression differences in *Drosophila* [Johnson et al., 2015, Noon et al., 2016]. Thus, future studies should address whether evolution of PREs might represent a mechanism to fuel differences in temporal *Hox* gene expression.

Hox genes are considered master regulators of numerous segment-specific morphologies and thus, they have been repeatedly implicated in the diversification of animal morphology [Carroll, 1995, Akam, 2002, Hughes and Kaufman, 2002]. Due to their pleiotropic nature, certain aspects of Hox-gene regulation may be more likely to undergo evolutionary changes. Compared to changes in Hox spatial expression, temporal shifts and their regulatory basis have been less explored. The discovery that Abd-B A5-A6 pupal expression is a derived state suggests the existence of underappreciated regulatory mechanisms contributing to temporal differences in Hox expression and morphological evolution.

4.0 Conclusion

Morphological evolution often results from differences in gene expression. Changes in the function of enhancers have been shown to greatly contribute to this process, the work presented here expands our understanding about the role of other kinds of regulatory elements. The function of silencers and time specific regulatory elements is crucial for achieving specific expression patterns. Here I showed that mutations affecting the activity of these regulatory elements can produce gene expression differences that contribute to the generation of morphological diversity.

Through the use of endogenous deletions I was able to show that silencers can repress redundant enhancers in a tissue-specific manner. However, it is still unclear whether all silencers share this property or if this is element-specific. In fact, it has been suggested that the mechanism of action of silencers may be variable. On the one hand silencers are thought to act on short distances silencing enhancers that are in close proximity; on the other hand, silencers might interact directly with promoters and recruit repressive proteins. Thus, only the second class might be able to act over redundant enhancers. Using chimeric reporter constructs and endogenous replacements would help to test these models. In fact, the deletion strains I created contain attP landing sites that could facilitate these experiments. Of great interest would be to replace the *D. melanogaster* silencers with silencers from other species and test whether they can repress the abdominal redundant enhancers.

An important question regarding the evolution of gene regulation is how a regulatory element, either an enhancer or a silencer, evolves in the first place. Three general scenarios can be predicted: 1) *de novo*, evolution from a non-functional DNA region; 2) co-option, evolution from a pre-existent regulatory element; 3) logic change, evo-

lution of an enhancer from a silencer, or vice versa. A great model for testing these models requires a recently evolved regulatory element. The midline silencer of *ebony* seems to have evolved exclusively in *D. melanogaster*. Even closely related species seem to lack this stripe of pigmentation. Moreover, this silencer shares sequence position with the upstream abdominal enhancer (an element which a highly conserved function). Thus, it is possible that the midline silencer might have evolved trough the co-option of the abdominal enhancer and, simultaneously, by changing regulatory logic. To test this hypothesis it would be interesting to clearly locate the boundaries between the enhancer and silencer elements and identify the regulators binding to each of these regions. This information could then be used to test specific evolutionary scenarios regarding the gain of a novel silencer.

The results presented in chapter two suggest that Abd-B regulation during pupal development is highly divergent even between closely related species. For instance, *D. ananassae* and *D. atripex* seem to have a functional pupal enhancer that, nevertheless is unable to drive Abd-B pupal expression in these species. This suggest that other changes might account for the lack of Abd-B pupal expression. The identification of such regulatory elements could expand our knowledge about *Hox* regulation. Interestingly, it is possible that Abd-B might be actively repressed during late developmental stages. Establishing techniques to monitor Abd-B mRNA during pupal development would be crucial to study the expression dynamics over time. Coupled with the deletion strains I generated this could help to identify DNA regions required for Abd-B silencing. Most of the work regarding *Hox* genes has focused on spatial repression. For instance, the *Mcp* boundary element is necessary for Abd-B repression in A4 and more anterior segments. Is this same element required for Abd-B repression during late developmental stages? The identification of such elements would help to better understand how Hox genes perform their function across space

and time. Likewise, it could be possible to test whether the lack of Abd-B pupal expression observed in some lineages is the result of active repression, which might have been secondarily lost in other lineages. Altogether, this work emphasizes the need to better characterize *Abd-B* temporal dynamics of expression. While I have identified two regulatory elements required for pupal expression, it is necessary to characterize the molecular mechanisms in charge of silencing this activity and I hope that the genetic resources generated in this work will help to achieve that.

Appendix Key resources, experimental models and methods details

A.1 *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). *D. melanogaster* lines were obtained from the Bloomington *Drosophila* stock center. 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. All strains used in Chpater 1 and 2 are listed in **Table 1** and **Table 5**, respectively.

A.2 CRISPR-*Cas9* genome editing

A.2.1 Donor vectors for homology 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). For *iab-5* deletions, the LHA was inserted into prVV661 (Addgene 108282) digested with SbfI-HF (NEB) and AvrII (NEB) via restriction cloning. The resulting plasmid was digested again with AscI (NEB) and KpnI (NEB) and the RHA was inserted via restriction cloning. The RFP marker

in prVV661 is flanked by attP sites, which allows for precise allele exchange using recombinase mediated cassette exchange (RMCE) [Voutev and Mann, 2018]. See **Table 2** and **Table 6**, for primers used in Chapter 1, and Chapter 2, respectively.

A.2.2 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 **Table 3** and **Table 7**, for primers used in Chapter 1, and Chapter 2, respectively.

A.2.3 *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, I 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 all deletions, I 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.4Δ and *iab-5* deletions were 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.

A.3 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). *D. melanogaster* transformant lines were generated by ΦC31 mediated site specific recombination into the 51D insertion site on the second chromosome. See **Table 4**, for primers used. 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.

A.4 Molecular cloning of plasmids used for RMCE

prVV578-xP3-GFP: The GFP coding sequence downstream the 3XP3 promoter and flanked by LoxP sites was amplified from pCRISPaint-3xP3-GFP (Addgene 130277) and inserted into prVV578 (Addgene 108279) digested with BamHI-HF (NEB) using NEBuilder DNA assembly (NEB). This plasmid contains attB sites flanking a multiple restriction cloning site and it was used for creating RMCE donor plasmids.

RMCE donors: The alleles used for replacements into *iab-5*Δ1 and *iab-5*Δ3 were amplified from genomic DNA of the targeted species and inserted into prVV578-3xP3-GFP digested with AscI (NEB) and NotI (NEB) using NEBuilder DNA assembly (NEB). See **Table 8**, for primers used.

A.5 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 $\times$ 100 [Rebeiz et al., 2009]. Boxplots were created using the R [R Core Team, 2022] packages ggplot2 [Wickham, 2016] and ggpubr [Kassambara, 2020].

A.6 Immunohistochemistry

Immunohistochemistry of the pupal abdominal epidermis was performed as previously described (19). Briefly, pupal abdomens at different developmental stages were dissected in cold PBS and fixed in PBS containing 4% paraformaldehyde (E.M.S. Scientific) and 0.1% Triton X-100 (PBT-fix). For species from the *melanogaster* subgroup, I used mouse monoclonal anti-Abd-B (Developmental Studies Hybridoma-Bank #1A2E9) 1:100 in PBT. For the other species, I used a custom rabbit polyclonal anti-Abd-B generated by genscript and diluted 1:10 in PBT. The polyclonal antibody was preadsorbed in *D. melanogaster* embryos by incubating overnight at 4 C. Primary incubation was performed overnight at 4 C for both antibodies and followed by three 15-minute PBT washes at room temperature. Secondary incubation was performed using Alexa Fluor 488 donkey anti-mouse and ait-rabbit IgG (InvitrogenThermo Fisher Scientific Life Technologies) 1:500 in PBT for 2.5 h at room temperature, followed by three 15-minutes washes in PBT. Samples were mounted

in glycerol mounting solution (80%glycerol, 0.1M Tris, pH 8.0) and imaged using a Leica SP8 CLARITY confocal microscope. Imaging settings were adjusted for each sample avoiding image saturation.

A.7 *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 (F - AGCAGCTTCTTCGACTAT, R - taatacgactcactatagggagaGCTTACAACTAGTCAACA). 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).

A.8 Key resources used in Chapter 2

Table 1: Wild type strains, deletion, and transgenic

Wild-type strains	**Source or reference**
Drosophila ananassae	#0000-1005.01
Drosophila malerkotliana	#14024–0391.00
Drosophila pseudoobscura	#0000-1006.01
Genetic reagents	**Source or reference**
D. melanogaster nos-Cas9(attP40)	BDSC 78781
D. melanogaster cre(III)	BDSC 1501
D. melanogaster double balancer	BDSC 3703
D. melanogaster $\Phi C31(X)$	BDSC 34772
D. melanogaster yw	Lab stock
D. melanogaster w	Lab stock
D. melanogaster eCRISPaint	e loss of function
D. melanogaster eActΔ	CRE deletion
D. melanogaster eActBΔ	CRE deletion
D. melanogaster eUpsΔ	CRE deletion
D. melanogaster eIn.4Δ	CRE deletion
D. melanogaster eMaleSilΔ	CRE deletion
D. melanogaster eStripeSilΔ	CRE deletion
D. melanogaster eActBΔ+In.4Δ	CRE deletion
D. melanogaster eUpsΔ+In.4Δ	CRE deletion
D. melanogaster ananassae_eAct	Transgenic reporter, inserted in 51D

D. melanogaster ananassae_eUS	Transgenic reporter, inserted in 51D
D. melanogaster ananassae_eUS+IN	Transgenic reporter, inserted in 51D
D. melanogaster malerkotliana_eAct	Transgenic reporter, inserted in 51D
D. melanogaster malerkotliana_eUS	Transgenic reporter, inserted in 51D
D. melanogaster malerkotliana_eUS+IN	Transgenic reporter, inserted in 51D
D. melanogaster malerkotliana_eUS+In.4	Transgenic reporter, inserted in 51D
D. melanogaster pseudoobscura_eAct	Transgenic reporter, inserted in 51D
D. melanogaster pseudoobscura_eUS	Transgenic reporter, inserted in 51D
D. melanogaster pseudoobscura_eUS+IN	Transgenic reporter, inserted in 51D
D. melanogaster pseudoobscura_eIN	Transgenic reporter, inserted in 51D
D. melanogaster melanogaster Ups	Transgenic reporter, inserted in 51D
D. melanogaster melanogaster Ups+IN	Transgenic reporter, inserted in 51D
D. melanogaster melanogaster Ups+In.1	Transgenic reporter, inserted in 51D
D. melanogaster melanogaster Ups+In.2	Transgenic reporter, inserted in 51D
D. melanogaster melanogaster Ups+In.3	Transgenic reporter, inserted in 51D

Table 2: Primers and plasmids used to clone homology directed repair donors to create *ebony* deletions. Left Homology Arm = LHA, Right Homology Arm = RHA.

Primer	**Construct**
tcttgcatgctagcggccgcACATTCCTCGCAATCCATGGAG	F - LHA eAct
gtgcatatgtccgcggccgcGGGTAAAAGTATGCACATATATTAGA	R - LHA eAct
ttaaatgcatgccctgcaggACATTCCTCGCAATCCATGGAG	F - LHA eActB

gcagatctaggcctcctaggTGAATTTGGCTTGGTGAAAGCAGG	R - LHA eActB
ttaaatgcatgccctgcaggCCTTCGCACCTATCGTAGCTAT	F - LHA eUps
cctaggaagcttcctgcaggCGCAGACCAGCACCAGACAT	R - LHA eUps
ttaaatgcatgccctgcaggGGAAACTGGATTCGCCTAACGG	F - LHA eIntron
cctaggaagcttcctgcaggCCCTGGCCTTAGCAAATGTTAATC	R - LHA eIntron
tcttgcatgctagcggccgcGCCACCACGCCTATCCATTAA	F - LHA eMaleSil
gtgcatatgtccgcggccgcCCATAAGCTGGTTTAATATCAGTT	R - LHA eMaleSil
ttaaatgcatgccctgcaggGGAAACTGGATTCGCCTAACGG	F - LHA eIn.4 (GFP)
taggtaccgtctcgagcagcTACCCTGGCCTTAGCAAATGTTAATC	R - LHA eIn.4 (GFP)
gtgcatatgtccgcggccgcCGAAGGAACTTAATTCGGCC	F - LHA eStripeSil
ctccatgcataaggcgcgccAAAGCCGTGCAGATGCAATG	R - LHA eStripeSil
ctccatgcataaggcgcgccATCAATTGAAGTGCTTAACAAATACC	F - RHA eAct
gcagaaggcctaggcgcgccCATCTGCGACCGTTTGTATCTG	R - RHA eAct
ccccagttgggggggcgcgccATCAATTGAAGTGCTTAACAAATACC	F - RHA eActB
aaagatcctctagaggtaccCATCTGCGACCGTTTGTATCTG	R - RHA eActB
ccccagttgggggggcgcgccATCCGGCGTCCACACACTGA	F - RHA eUps
catatggctagcggcgcgccCAAGTGCCTTACGTCAATGGG	R - RHA eUps
ccccagttgggggggcgcgccCCTAGGGATTCTCCGACTGAG	F - RHA eIntron
catatggctagcggcgcgccCTCTTCTCCACAGCTAAAGGATTC	R - RHA eIntron
ctccatgcataaggcgcgccTACTGGCGTTGATTCTTGTACG	F - RHA eMaleSil
gcagaaggcctaggcgcgccCAGCATACCACACATACCCA	R - RHA eMaleSil
gtgagggttaattgcgcgctCCTAGGGATTCTCCGACTGAG	F - RHA eIn.4 (GFP)
catatggctagcggcgcgccCTCTTCTCCACAGCTAAAGGATTC	R - RHA eIn.4 (GFP
gtgcatatgtccgcggccgcCGAAGGAACTTAATTCGGCC	F - RHA eStripeSil
ctccatgcataaggcgcgccAAAGCCGTGCAGATGCAATG	R - LHA eStripeSil

AAGGATTTCTCGCTACGCAC	F - sequence confirmation eActΔ
GCCCGTTCCATAAGGATACATCGCAA	F - sequence confirmation eUpsΔ
GGCGCGATTAGAAGACCAGTTTTTCT	F - sequence confirmation eIn.4Δ
CCCCGATCAATTGAAGTGCT	F - sequence confirmation eMaleSilΔ
CAACAGTGAATGCAACAAGCG	F - sequence confirmation eStripeSilΔ
AAAGAAGGTGTGTCCCAGGT	R - sequence confirmation eActΔ
GTTGAAAGACCGTTTGCCAGTGGC	R - sequence confirmation eUpsΔ
GCCGTTAGCTTTAGATCCTCAGGAAC	R - sequence confirmation eIn.4Δ
AACTCAACGTGCTATGTACTTG	R - sequence confirmation eMaleSilΔ
CTTAAGCTGCAGAGATTGCAAG	R - sequence confirmation eStripeSilΔ
GATCCCGTACGATAACTTCG	R - sequencing the location of the dsRed marker

TCGCGGGAAGTTCCTATACCTT	R - sequencing the location of the RFP marker
TTCACTGCATTCTAGTTGTGGT	F - sequencing the location of the dsRed marker
GGTATAGGAACTTCACCG	F - sequencing the location of the RFP marker
Plasmid	**Source or reference**
phD-dsRed	Addgene #51434
prVV661	Addgene # 108282

Table 3: Primers used to synthesize sgRNAs targeting *ebony* regulatory regions. Left Homology Arm = LHA, Right Homology Arm = RHA.

Primer	**Target**
AAAAAAAGCACCGACTCGGTGCCACTTTTT	sgRNA backbone - overlap PCR
CAAGTTGATAACGGACTAGCCTTATTTTAA	
CTTGCTATTTCTAGCTCTAAAAC	
AAAATAATACGACTCACTATAGG	T7-F - overlap PCR

AAAAAAGCACCGACTCGGTGCCA	sgRNA-backbone-R - overlap PCR
aaaataatacgactcactatagg()gttttagagctag	() replaced with target sequence - overlap PCR
GCCGGGAAGCTGGGATCGAT	sgRNA targeting the first *ebony* exon
ACTTCCATAGCCGTTACTTG	sgRNA targeting the LHA of eAct
TTCCATAGCCGTTACTTGAG	sgRNA targeting the LHA of eAct
GTTAAGCACTTCAATTGATC	sgRNA targeting the LHA of eActB
TTAAGCACTTCAATTGATCG	sgRNA targeting the LHA of eActB
CGATAAGGATTAGTAATATA	sgRNA targeting the LHA of eUps
TCTGGTGCTGGTCTGCGATA	sgRNA targeting the LHA of eUps
TAAGGCCAGGGGTATGGCAC	sgRNA targeting the LHA of eIn.4
AATTACCCGTGCCATACCCC	sgRNA targeting the LHA of eIn.4
ATTTGCTAAGGCCAGGGGTA	sgRNA targeting the LHA of eIn.4

ATTAAACCAGCTTATGGTTG	sgRNA targeting the LHA of eMaleSil
ATTGGGCCACAACCATAAGC	sgRNA targeting the LHA of eMaleSil
ATATACGAAGGAACTTAATT	sgRNA targeting the LHA of eStripeSil
GTAAGTTTCGTTATATACGA	sgRNA targeting the LHA of eStripeSil
GTTAAGCACTTCAATTGATC	sgRNA targeting the RHA of eAct
TTAAGCACTTCAATTGATCG	sgRNA targeting the RHA of eAct
AAGCGGTACCAATGCTAATC	sgRNA targeting the RHA of eUps
TGTGGACGCCGGATTAGCAT	sgRNA targeting the RHA of eUps
GGGAATGATGATGTAACCTA	sgRNA targeting the RHA of eIn.4
TGGGAATGATGATGTAACCT	sgRNA targeting the RHA of eIn.4
GCATCGCAATTTGGATTTAC	sgRNA targeting the RHA of eMaleSil
TTGCATCTGCACGGCTTTGT	sgRNA targeting the RHA of eStripeSil

TGCATCTGCACGGCTTTGTT	sgRNA targeting the RHA of eStripeSil

Table 4: Primers used to clone *ebony* transgenic reporters into the plasmid S3aG.

Primer	**Construct**
agcccgggcgaattcgccggcgcgccAAGCGCTGGTAATCCTGC	F - *D. ananassae* eAct
ggttgcgatcgcttcctgcaggGTTGGACATGACAATGACAGCG	R - *D. ananassae* eAct
agcccgggcgaattcgccggcgcgccAAGCGCTGGTAATCCTGC	F - *D. ananassae* eUps
ggttgcgatcgcttcctgcaggCCAGTTCCTGCTCTTAAA	R - *D. ananassae* eUps
atcaatgtatcttaactagtCTTGGCATGCCTAATAAC	F - D. *ananassae* eUps+IN
acacttattacgtgactagtCATTGCATCATTCTTGGG	R - *D. ananassae* eUps+IN
agcccgggcgaattcgccggcgcgccAAGCGCTGGTAATCCTGC	F - *D. malerkotliana* eAct
ggttgcgatcgcttcctgcaggGTTGGACATGACAATGACAGCG	R - *D. malerkotliana* eAct

agcccgggcgaattcgccggcgcgccAAGCGCTGGTAATCCTGC	F - *D. malerkotliana* eUps
ggttgcgatcgcttcctgcaggCCAGTTCCTGCTCTTAAA	R - *D. malerkotliana* eUps
atcaatgtatcttaactagtCTTGGCATGCCTAATAAC	F - *D. malerkotliana* eUps+IN
acacttattacgtgactagtCATTGCATCATTCTTGGG	R - *D. malerkotliana* eUps+IN
agcccgggcgaattcgccggcgcgccCGCGTGCTCGTTGATAAG	F - *D. pseudoobscura* eAct
ggttgcgatcgcttcctgcaggAACATCCGGACTTCCCCAAAGA	R - *D. pseudoobscura* eAct
agcccgggcgaattcgccggcgcgccCGCGTGCTCGTTGATAAG	F - *D. pseudoobscura* eUps
ggttgcgatcgcttcctgcaggCAGTTCCTGCTCTTACAG	R - *D. pseudoobscura* eUps
atcaatgtatcttaactagtGACTTGACATGCATGTTG	F - *D. pseudoobscura* eUps+IN
acacttattacgtgactagtTTCTTGGGGTTAGTTAGG	R - *D. pseudoobscura* eUps+IN
atcaatgtatcttaactagtCTTGGCATGCCTAATAAC	F - *D. malerkotliana* eUps+In.4
acacttattacgtgactagtATTCGTTACGTATACGCCCCGT	R - *D. malerkotliana* eUps+In.4

AACTCGCTTTCCCGAAATTAATGTGC	F - *D. melanogaster* eUps+In.1
TTGTGAGTCAATTGGATGACAAGC	R - *D. melanogaster* eUps+In.1
TTGAGTCATTGGCCAAAGCGATCG	F - D. *melanogaster* eUps+In.2
ATTACGTATGCGCCGTGTGAGTCC	R - *D. melanogaster* eUps+In.2
TCTAACGCAAATGCAACCTTG	F - *D. melanogaster* eUps+In.3
AATTGAGATGTGTTCCGGCTT	R - *D. melanogaster* eUps+In.3
ACGAGGCCTCAAATCTAATGAAATC	F - *D. melanogaster* eUps+In.4
TTGGGCTTAGAATCTCAGTCGGAGAA	R - *D. melanogaster* eUps+In.4

A.9 Key resources used in Chapter 3

Table 5: Wild type strains, deletion, and replacement lines.

Wild-type strains	**Source or reference**
Drosophila yakuba	#14021-0261-01
Drosophila santomea	#14021-0271.00

Drosophila auraria	#14028-0471.00
Drosophila ananassae	#0000-1005.01
Drosophila atripex	#14024-0361.00
Drosophila willistoni	#14030-0811.24
Genetic reagents	**Source or reference**
D. melanogaster nos-Cas9(attP40)	BDSC 78781
D. melanogaster cre(III)	BDSC 1501
D. melanogaster double balancer	BDSC 3703
D. melanogaster $\Phi C31(X)$	BDSC 34772
D. melanogaster yw	Lab stock
D. melanogaster w	Lab stock
D. melanogaster iab-5Δ1	CRE deletion
D. melanogaster iab-5Δ1	CRE deletion
D. melanogaster iab-5Δ3	CRE deletion
D. melanogaster iab-5Δ4	CRE deletion
D. melanogaster iab-5Δ1A	CRE deletion
D. melanogaster iab-5Δ1B	CRE deletion
D. melanogaster iab-5Δ1C-	CRE deletion
D. melanogaster iab-5Δ1+mel	CRE replacement
D. melanogaster iab-5Δ1+yak	CRE replacement
D. melanogaster iab-5Δ1+san	CRE replacement
D. melanogaster iab-5Δ1+aur	CRE replacement
D. melanogaster iab-5Δ1+ana	CRE replacement
D. melanogaster iab-5Δ1+atrip	CRE replacement
D. melanogaster iab-5Δ1+pseu	CRE replacement

D. melanogaster iab-5Δ1+wil	CRE replacement
D. melanogaster iab-5Δ3C-	CRE deletion
D. melanogaster iab-5Δ3+mel	CRE replacement
D. melanogaster iab-5Δ3+yak	CRE replacement
D. melanogaster iab-5Δ3+san	CRE replacement
D. melanogaster iab-5Δ3+aur	CRE replacement
D. melanogaster iab-5Δ3+pseu	CRE replacement
D. melanogaster iab-5Δ3+wil	CRE replacement

Table 6: Primers and plasmids used to clone homology directed repair donors to create *iab-5* deletions. Left Homology Arm = LHA, Right Homology Arm = RHA.

Primer	**Construct**
ttaaatgcatgccctgcaggCTTGGCCGTGGTCGTTTTTT	F - LHA iab-5.1
gcagatctaggcctcctaggTCTTGGTATTGGGTAAAGAA	R - LHA iab-5.1
aaaGGCGCGCCACTTGGTCGACGGAGGCG	F - LHA iab-5.1
aaaGGTACCCTACCAACTTCGCATCGGAT	R - LHA iab-5.1
aaaCCTGCAGGGCGGCCAGATAAAAGTGGTC	F - LHA iab-5.2
aaaCCTAGGGCCCAGGTATCTCCAAGCAA	R - LHA iab-5.2
aaaGGCGCGCCTACAAATCCGGACGCAGCCT	F - LHA iab-5.2
aaaGGTACCATGTCAAGTCGCTGAGATCG	R - LHA iab-5.2
aaaCCTGCAGGGCAGCAATCGAAGGAAACAG	F - LHA iab-5.3
aaaCCTAGGCTGGTCAGTAAACGGGTCCC	R - LHA iab-5.3

aaaGGCGCGCCGAGGGGAGGGTGGGGCAGAA	F - LHA iab-5.3
aaaGGTACCCCGACATTGTATCTGTGTGACG	R - LHA iab-5.3
aaaCCTGCAGGTCCTCTGGTCCTGAAACTCT	F - LHA iab-5.4
aaaCCTAGGCGTCGGAGGTCTTAATTTAAACTG	R - LHA iab-5.4
aaaGGCGCGCCTCAGGGGTAACAAAGCGCAA	F - LHA iab-5.4
aaaGGTACCTGTGTTTTGGACGGGACCAC	R - LHA iab-5.4
TTTATATCCACTGTGCGGCG	F - sequence confirmation iab-5.1
CCCTGTTTCCTTCGATTGCT	R - sequence confirmation iab-5.1
TGCATTGTGAGAAATCGGGG	F - sequence confirmation iab-5.2
TCGTTGCCATCCGTGTCTTA	R - sequence confirmation iab-5.2
TGGTAGCGAATGGCGACATT	F - sequence confirmation iab-5.3
AACATTGATGGCTGCCTCTG	R - sequence confirmation iab-5.3
GAAACGATAACGAAACGGGG	F - sequence confirmation iab-5.4
CTGCCGAATTTCACTATTGG	R - sequence confirmation iab-5.4
AAGCTTCCTAGGAGGCCTAG	F - sequencing the location of the RFP marker

GTCGCCACCAATCCCCATAT	R - sequencing the location of the RFP marker
Plasmid	**Source or reference**
prVV661	Addgene # 108282

Table 7: Primers used to synthesize sgRNAs targeting *iab-5* regions. Left Homology Arm = LHA, Right Homology Arm = RHA.

Primer	**Target**
AAAAAAAGCACCGACTCGGTGCCACTTTTT CAAGTTGATAACGGACTAGCCTTATTTTAA CTTGCTATTTCTAGCTCTAAAAC	sgRNA backbone - overlap PCR
AAAATAATACGACTCACTATAGG	T7-F - overlap PCR
AAAAAAGCACCGACTCGGTGCCA	sgRNA-backbone-R - overlap PCR
aaaataatacgactcactatagg()gttttagagctag	() replaced with target sequence - overlap PCR
TGGAACGATTACGAAAGTCT	sgRNA targeting the LHA of iab-5.1
GATTACGAAAGTCTTGGTAT	sgRNA targeting the LHA of iab-5.1
CTTCCGAACTTGGTCGACGG	sgRNA targeting the RHA of iab-5.1

AGCCATTCCACTTCCGAACT	sgRNA targeting the RHA of iab-5.1
GCCGATTGAACCAATTGCCC	sgRNA targeting the LHA of iab-5.2
CTTGGAGATACCTGGGCAAT	sgRNA targeting the LHA of iab-5.2
GTTTCCAGGGTTTACAAATC	sgRNA targeting the RHA of iab-5.2
GCGTCCGGATTTGTAAACCC	sgRNA targeting the RHA of iab-5.2
TTGCAACTCACTTCACCTTC	sgRNA targeting the LHA of iab-5.3
GGACCCGTTTACTGACCAGA	sgRNA targeting the LHA of iab-5.3
TGCCCCACCCTCCCCTCGAT	sgRNA targeting the RHA of iab-5.3
GCAGTAGGCAGGGCCAATCG	sgRNA targeting the RHA of iab-5.3
ATTAATATAAACAAGAACGT	sgRNA targeting the LHA of iab-5.4
AATATAAACAAGAACGTCGG	sgRNA targeting the LHA of iab-5.4
CGCTTTGTTACCCCTGAAAA	sgRNA targeting the RHA of iab-5.4

GCTTTGTTACCCCTGAAAAT	sgRNA targeting the RHA of iab-5.4

Table 8: Primers and plasmids used to clone RMCE donors to create *iab-5* replacements used in Chapter 3.

gccatatgggtaccggatccTAGCTGCTCGAGACGGTACCTA	F - inserting GFP into prVV578
ggctccccgggcgcggatccAGCGCGCAATTAACCCTCAC	Reverse -inserting GFP into prVV578
AGGCCTAGATCTgcggccgcCCATTGGAATGGAGACTCGC	F - RMCE donor iab-5Δ1A
CATATGGCTAGCggcgcgccAAGTCCCTACAGTCTGCTGG	R - RMCE donor iab-5Δ1A
AGGCCTAGATCTgcggccgcTTGGCCAACACTTTCCTCGTC	F - RMCE donor iab-5Δ1B
CATATGGCTAGCggcgcgccTCGGAAGTGGAATGGCTTCTG	R - RMCE donor iab-5Δ1B
aacgcgtgaatgggcgcgccCCAGCTAATTGACAATTAGCTGACA	F - RMCE donor iab-5Δ1+mel, yak, san
aacgcgtgaatgggcgcgccCCAGCTAATTGACAATTAGCGGACA	F - RMCE donor iab-5Δ1+aur
aacgcgtgaatgggcgcgccCCAGCTAATTGACAATTAGCCACAA	F - RMCE donor iab-5Δ1+ana, atrip

aacgcgtgaatgggcgcgccCCAGCTAATTGACAATTAGCGGGC	F - RMCE donor iab-5Δ1+pseu
aacgcgtgaatgggcgcgccGGCTAATTGACACGACAATTGAGCG	F - RMCE donor iab-5Δ1+wil
catatggctagcggcgcgccGGAATGGCTTCTGTCTGCTGATA	R - RMCE donor iab-5Δ1+mel, yak, san, aur
catatggctagcggcgcgccTTCCTCCAATGGCTTCTGTCTG	R - RMCE donor iab-5Δ1+ana, atrip
catatggctagcggcgcgccGGGTTCATTATGGAAATGGCTTCT	R - RMCE donor iab-5Δ1+pseu
catatggctagcggcgcgccGGTTGGAGCATAACAACAACGAAG	R - RMCE donor iab-5Δ1+wil
aacgcgtgaatgggcgcgccAATCCGGACGCAGCCTTGCA	F - RMCE donor iab-5Δ3+mel
aacgcgtgaatgggcgcgccAATCCGGACGCAGCTTTGCATT	F - RMCE donor iab-5Δ3+yak, san
aacgcgtgaatgggcgcgccCTGGCCTTCTGCAGTTCTGC	F - RMCE donor iab-5Δ1+aur
aacgcgtgaatgggcgcgccGACGCAGCTCTGCATTTTACAAT	F - RMCE donor iab-5Δ1+pseu
aacgcgtgaatgggcgcgccCCTCCTTCCATCGTTCTTATCC	F - RMCE donor iab-5Δ1+wil
catatggctagcggcgcgccTAAACAAGAACGTCGGAGGTCTTA	R - RMCE donor iab-5Δ3+mel

catatggctagcggcgcgccTAAACAAGAACGTCGGGACTCTTA	R - RMCE donor iab-5Δ3+yak, san
catatggctagcggcgcgccAATGTTGGGGACAGCAGGAG	R - RMCE donor iab-5Δ1+aur
catatggctagcggcgcgccTTCAGCTGAACGCGACTTGTAC	R - RMCE donor iab-5Δ1+pseu
catatggctagcggcgcgccCAGTGAATTCCTATGAGGACTTTTC	R - RMCE donor iab-5Δ1+wil
CACTTCAAGCTCGCTTTGAAATACGC	F - confirming replacements into iab-5Δ1
CCCAATACCAAGACTTTCGTAATCG	F - confirming replacements into iab-5Δ1
GCCAAGAGTTCCATCCCATTTTGA	R - confirming replacements into iab-5Δ1
AGAAATGTGTTCGTATGCGATCGC	R - confirming replacements into iab-5Δ1
CGGTCCCTGGATGCGTTTCATTAA	F - confirming replacements into iab-5Δ3

GGTCTAAATCAATCTGCGGCACCT	R - confirming replacements into iab-5Δ3
CCGGTACCCATATGGCTAGC	prVV578 internal primer to confirm replacements
GTCTCGAGCAGCTAGGATCC	prVV578 internal primer to confirm replacements
Plasmid	**Source or reference**
prVV578	Addgene # 108279
prVV578+GFP	This study

A.10 Allele replacements using Recombinase Mediated Cassette Exchange

Recombinase Mediated Cassette Exchange (RMCE) was performed following Voutev and Mann [2018] with slight modifications. RMCE consists of two steps. First, the desired DNA region is deleted using CRISPR-*Cas9* and attP sites for recombination are introduced to create an RMCE platform line. Second, the recombinant allele is introduced in the platform line using ϕC31. RMCE can be performed using a donor plasmid that lacks a marker and identifying transformants by lack of RFP eye expression. In my experience, screening in this way can be challenging since deletions lines (like *iab-5*) sometimes have low RFP expression. Thus, I decided to

use a positive marker (3xP3-GFP). This makes the screening process easier but it requires an extra step to remove the GFP marker and obtain a clean replacement. **Fig. 32** illustrates this process.

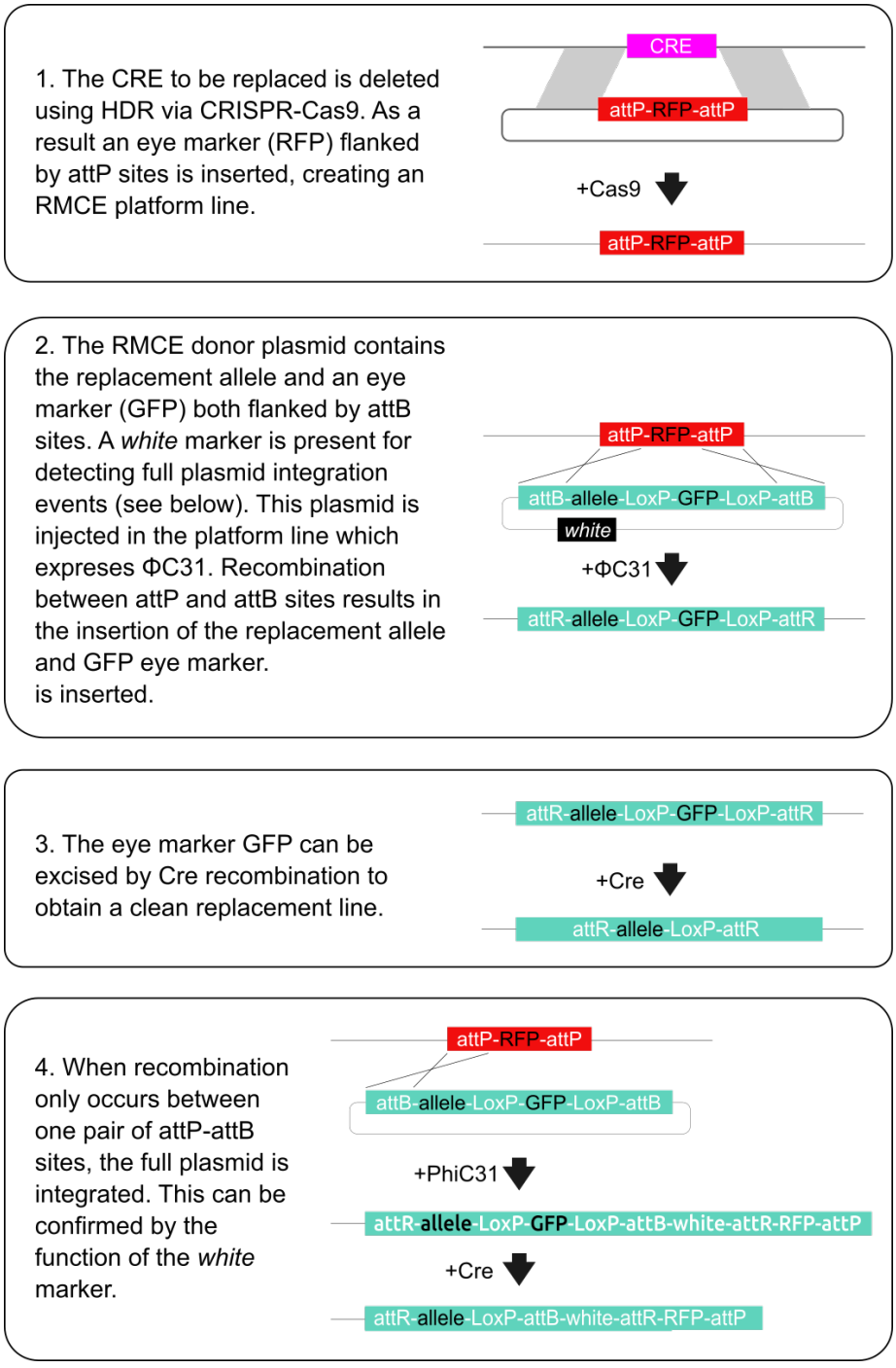

Figure 32: CRE replacement using RMCE

Here I show the crosses used to perform RMCE. First, a *nos*-ΦC31 transgene was introgressed on the X chromosome of the RMCE platform line marked by 3xP3-RFP. This line was injected with a RMCE donor plasmid marked by 3xP3-GFP flanked by LoxP sites, and w^+ as a marker for plasmid integration events. Thus, recombination events are identified by RFP- and GFP+ flies (the latter can be excised using Cre).

1. Cross virgins from the integrase line (Bloomington ID 34772) with your modified line:

$$\frac{y,w,y^+[\Phi C31]}{y,w,y^+[\Phi C31]};\ \frac{L}{cyo};\ \frac{TM2}{TM6B}\quad \mathbf{X}\quad \frac{w}{Y};\ \frac{+}{+};\ \frac{RFP}{RFP}$$

2. Collect males *cyo*, RFP/TM6B and cross with virgins from the integrase line:

$$\frac{y,w,y^+[\Phi C31]}{y,w,y^+[\Phi C31]};\ \frac{L}{cyo};\ \frac{TM2}{TM6B}\quad \mathbf{X}\quad \frac{y,w,y^+[\Phi C31]}{Y};\ \frac{+}{cyo};\ \frac{RFP}{TM6B}$$

3. Collect males and females *cyo*, RFP/TM6B and cross with each other:

$$\frac{y,w,y^+[\Phi C31]}{y,w,y^+[\Phi C31]};\ \frac{+}{cyo};\ \frac{RFP}{TM6B}\quad \mathbf{X}\quad \frac{y,w,y^+[\Phi C31]}{Y};\ \frac{+}{cyo};\ \frac{RFP}{TM6B}$$

4. Select against *cyo* and *TM6B* and cross siblings:

$$\frac{y,w,y^+[\Phi C31]}{y,w,y^+[\Phi C31]};\ \frac{+}{+};\ \frac{RFP}{RFP}\quad \mathbf{X}\quad \frac{y,w,y^+[\Phi C31]}{Y};\ \frac{+}{+};\ \frac{RFP}{RFP}$$

Amplify line, set chamber and inject embryos

5. After injections, adults will be crossed with *yw*:

$$(injected\ male)\quad \frac{yw}{yw};\ \frac{+}{+};\ \frac{+}{+}\quad \mathbf{X}\quad \frac{y,w,y^{+}[\Phi C31]}{Y};\ \frac{+}{+};\ \frac{\textbf{RFP-,GFP?}}{RFP}$$

$$(injected\ female)\quad \frac{y,w,y^{+}[\Phi C31]}{y,w,y^{+}[\Phi C31]};\ \frac{+}{+};\ \frac{\textbf{RFP-,GFP?}}{RFP}\quad \mathbf{X}\quad \frac{yw}{Y};\ \frac{+}{+};\ \frac{+}{+}$$

Full recombination events will produce w GFP progeny. Plasmid integration events will produce w^{+} GFP RFP progeny. If only integration events are obtained, keep the integrase on the background for another generation. This can lead to intra chromosomal recombination and the deletion of the w^{+} marker and vector backbone. However, the efficiency of this may be low.

6. Select GFP males and cross with virgins from a 3rd chromosome balancer line

$$\frac{w}{w};\ \frac{+}{+};\ \frac{MKRS}{TM6B}\quad \mathbf{X}\quad \frac{yw}{Y};\ \frac{+}{+};\ \frac{\textbf{GFP}}{+}$$

7. Select GFP/TM6B males and remove the GFP marker by crossing with virgins from a Cre line (Bloomington 1501)

$$\frac{y,w}{y,w};\ \frac{+}{+};\ \frac{MKRS,y^{+}[FLP]}{TM6B,w^{+}[Cre]}\quad \mathbf{X}\quad \frac{w}{Y};\ \frac{+}{+};\ \frac{\textbf{GFP}}{TM6B}$$

8. Select males that are not MKRS, which will be w^{+} and have the GFP marker removed (i.e., GFP-/TM6B,w^{+}[Cre]) and cross with virgins from a 3rd chromosome balancer line

$$\frac{w}{w};\ \frac{+}{+};\ \frac{MKRS}{TM6B} \quad \mathbf{X} \quad \frac{yw}{Y};\ \frac{+}{+};\ \frac{\mathbf{GFP\text{-}}}{TM6B, w^{+}[Cre]}$$

9. Select males that are not MKRS and cross with virgins from 3rd chromosome balancer line

$$\frac{w}{w};\ \frac{+}{+};\ \frac{MKRS}{TM6B} \quad \mathbf{X} \quad \frac{w}{Y};\ \frac{+}{+};\ \frac{\mathbf{GFP\text{-}}}{TM6B}$$

10. Cross siblings that are not MKRS

$$\frac{w}{w};\ \frac{+}{+};\ \frac{\mathbf{GFP\text{-}}}{TM6B} \quad \mathbf{X} \quad \frac{w}{Y};\ \frac{+}{+};\ \frac{\mathbf{GFP\text{-}}}{TM6B}$$

11. Cross siblings that are not TM6B (WT pupae is easier). **Note:** Recombination can occur in both directions, thus it is important to sequence the region and determine the direction of the recombined allele.

$$\frac{w}{w};\ \frac{+}{+};\ \frac{\mathbf{GFP\text{-}}}{\mathbf{GFP\text{-}}} \quad \mathbf{X} \quad \frac{w}{Y};\ \frac{+}{+};\ \frac{\mathbf{GFP\text{-}}}{\mathbf{GFP\text{-}}}$$

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