Using *Drosophila* pigmentation traits to study the mechanisms of *cis*-regulatory evolution

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Abstract

One primary agenda of the developmental evolution field is to elucidate molecular mechanisms governing differences in animal form. While mounting evidence has established an important role for mutations in transcription controlling *cis*-regulatory elements (CREs), the underlying mechanisms that translate these alterations into differences in gene expression are poorly understood. Emerging studies focused on pigmentation differences among closely related *Drosophila* species have provided many examples of phenotypically relevant CRE changes, and have begun to illuminate how this process works at the level of regulatory sequence function and transcription factor binding. We review recent work in this field and highlight the conceptual and technical challenges that currently await experimental attention.

Introduction

Many insights into the molecular events that drive the appearance of complex organismal traits have come from integrating developmental genetics into the study of evolution [1]. Multicellular animals and their distinguishing features are built during the course of development, and by examining developmental processes, one can determine how such mechanisms change. A key driver of development is differential gene activity: the specification of which subset of the genome is activated, or expressed at each step of the developmental sequence. Work in the developmental evolution field over the last several years has established an important role for transcriptional regulatory DNA mutations in altering gene expression patterns and thereby phenotypic traits [2–8]. As the molecular details of *cis*-regulatory mutations emerge, we can compose a deeper mechanistic understanding of complex evolutionary problems such as trait origination, coordination, pleiotropy, and phenotypic plasticity. Here we review how recent work on fruit fly pigmentation has contributed to the current view of developmental evolution, and highlight the challenges and rewards for addressing these systems in more sophisticated mechanistic detail.

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The *Drosophila* pigmentation model

The pigmentation patterns decorating the bodies of fruit flies are remarkably diverse [9–12]. These patterns have been associated with various aspects of organismal fitness, such as resistance to desiccation [13,14], ultra violet radiation protection [15,16], and courtship success [17,18]. The genetic composition for three pigmentation traits in particular has received considerable experimental attention (Figure 1). In the abdomen of *D. melanogaster,* male-specific pigmentation on the entire A5 and A6 tergites evolved from an ancestor that displayed only thin stripes of pigment along each tergite. The male-specific spot on the antero-distal region of the *D. biarmipes* wing, and the spotted pattern adorning the wings of *D. guttifera* both evolved from ancestors that produced uniformly unpigmented wings. Several of the genes that must be specifically expressed to generate these pigmentation patterns are now known, and many details of their transcriptional regulation have been recently characterized.

Each pigmentation feature is associated with the patterned expression of enzymes that produce melanin pigments. The gene *yellow* is expressed in zones where dark melanic pigmentation occurs on the cuticle [19–21]. While several hypotheses exist about the molecular function of *yellow* [22], its expression appears to be a general requirement for black melanin formation. For the three traits considered here (Figure 1), there also exists a reciprocal exclusion of *ebony* gene expression [8,19,23]. *ebony* encodes an enzyme that converts Dopamine into N-ß-alanyl dopamine (NBAD), which generates yellow-colored cuticle [24]. While other pigmentation enzymes contribute to cuticle coloration, only *ebony* and *yellow* have thus far been associated with strong expression patterns that predict the wing phenotypes. For male-specific abdomen pigmentation, an additional gene, *tan,* is expressed in a pattern which closely matches that of *yellow* [21]. *tan* encodes an enzyme with NBAD hydrolase activity which converts NBAD into Dopamine, promoting the formation of darker cuticle pigments [25].

Many of the upstream regulators responsible for the patterns of *yellow*, *tan* and *ebony* expression have been identified. In the abdomen, the Hox gene *Abd-B* is expressed in the epidermis underlying the pigmented A5 and A6 tergites and is required for the activation of *yellow* and *tan* [21,26]. The expression of *yellow* and *tan* in the unpigmented female abdomen is prevented by the Bab1 and Bab2 transcriptional repressors [27]. In the wing of *D. biarmipes*, the transcription factor Distalless (Dll) has been identified as a major input mediating activation and mutual exclusion of *yellow* and *ebony* respectively [28]. The wing spots of *D. guttifera* were associated with the specific expression of the *wingless* (*wg*) morphogen gene. RNAi screens of the majority of transcription factors in the *D. melanogaster* genome have revealed dozens of additional factors required for pigmentation phenotype and/or gene activity in the wing and abdomen [28,29] whose direct connections to the regulatory regions of pigmentation genes await elucidation.

Regulatory control of the *Drosophila* pigmentation genes

The expression of each pigmentation gene during development is governed by at least one *cis*-regulatory element (**CRE**) sequence that controls its timing and spatial extent of transcription. CREs often extend over a hundred base pairs in length and include short 5-20

base pair motifs that function as **binding sites** for a specific set of transcription factors that form their **regulatory logic**. The main pigmentation gene CREs have been defined, and a portion of their regulatory logic has been elucidated. For the *tan* and *yellow* genes, single CREs known as the *tan* Male Specific Element or "t_MSE" [21,30] and the *yellow* Body Element or "yBE" [7,26] drive their male-limited abdominal patterns respectively. *ebony* on the other hand requires the activity of an activating enhancer and two repressive silencer elements to produce a reciprocal pattern of expression in the *D. melanogaster* abdomen [23]. For wing spots, *yellow* is the only gene for which CREs have been characterized. In *D. biarmipes*, an antero-distal spot of expression in the pupal wing is driven by a CRE known as the spot element [8]. In *D. guttifera*, the 16 spots of expression are driven by a CRE known as "vein spot" [20]. The identification of these CRE architectures has facilitated mechanistic studies of several complex evolutionary processes, such as the origination, coordination, and constraint of their developmental programs.

Elucidating the origins of pigmentation traits and the CREs that drive them

One way to study the origin of a trait is to examine the evolutionary history of the CREs that drive patterned expression of key trait-building genes. For example, in a seminal study of the wing spot of *D. biarmipes*, it was found that the *yellow* spot element evolved upon a CRE that ancestrally functioned to direct a low level of expression across the entire wing epidermis [8]. The origin of the spot element occurred through the co-option of Dll activation and En repression which required the evolution of binding sites for these two transcription factors in the spot CRE (Figure 2) [28]. Regarding the 16 pigmentation spots in *D. guttifera*, the origin of the vein spot CRE appears to have involved the evolution of connections to the Wingless signaling pathway through binding inputs that still await identification [20]. Recent dissection of CREs in the *wg* locus has revealed that its expression in four vein tips evolved in the locality of a conserved enhancer active in the crossveins of diverse species, including *D. melanogaster* [31]. In contrast, a CRE that drives five spot-associated foci of *wg* expression in cells of the campaniform sensilla evolved in the intron of the nearby *Wnt10* gene, where no CRE activity has yet been characterized. These findings demonstrate how new CREs are associated with new expression patterns and traits, and may often originate through the co-option of pre-existing activities.

The full tergite pigmentation of the *D. melanogaster* male posterior abdomen (Figure 1E) evolved from an ancestor that lacked this trait, as is observed in current day *D. willistoni* (Figure 1D) [26]. Tests of the yBE and t_MSE from *D. willistoni* revealed an inability to drive expression in the abdomen [21]. This supports a model whereby the origin of this trait required the evolution of these CREs. Within the yBE and t_MSE, a regulatory logic evolved that at a minimum involved direct binding of Abd-B to the yBE, and connected Abd-A and Hth to the t_MSE. Furthermore, additional inputs for sex-specific expression must have formed at both CREs (Figure 3). At present, these studies cannot resolve whether these evolutionary transitions occurred through the *de novo* evolution of a functional CRE or by the repurposing of an ancestral CRE, as was the case for the spot and vein spot/tip CREs. In the case of *yellow,* this CRE evolved next to a CRE that produces a thin stripe of expression at the posterior edges of tergites, raising the possibility that this activity may have been co-opted [21], but this model warrants further examination. Collectively, each of these cases of

trait origination was accompanied by the insertion of one or more transcription factors into the regulatory logic of a differentiation gene CRE. However, the coordinated evolution of co-expressed CRE activities can arise by evolving very different regulatory inputs.

The evolution of coordinately regulated expression programs

An impressive degree of orchestration underlies the hundreds of gene expression decisions that mark the progression of development. Pigmentation traits provide excellent models to address how the coordinated deployment of multiple genes evolves to alter a phenotype. In the case of *D. biarmipes*, several closely related species possess spots of different shapes and intensities, and the patterns of Yellow expression closely pre-configures the adult pattern of pigmentation [28]. In each species tested, the pattern of Dll expression closely matches the pattern of the adult wing pigmentation pattern (Figure 2). This suggests that upon the origin of the Dll-regulated pigmentation spot, diversity has been largely shaped by evolving patterns of Dll expression. However, it is currently not known whether the causative mutations reside within the *Dll* locus, or alternatively reside in an upstream regulator of *Dll* expression in the wing.

The sexually dimorphic abdominal pigmentation of *D. melanogaster* and its relatives provide a particularly convenient model to study the coordinated evolution of a gene expression program. Within the *melanogaster* species group, tergite pigmentation ranges from the A6 segment in species like *D. auraria* (Figure 1F), the A5 and A6 segments of species such as *D. melanogaster* (Figure 1E), to the expanded A3-A6 segment pigmentation found in *D. prostipennis* (Figure 1G). In *D. prostipennis*, *yellow* and *tan* expression has expanded beyond A5 and A6 to include the A3 and A4 segments, and likewise *ebony* expression has receded from these segments [32]. The CREs for these three genes were tested for their ability to regulate GFP reporter expression in transgenic *D. melanogaster*. For the yBE CRE, the *D. prostipennis* reporter recapitulated the male expression of *yellow* in the A3-A6 segments indicating *cis*-evolutionary change at this CRE. However, the t_MSE and *ebony* CREs drove patterns of GFP expression indistinguishable from those driven by the *D. melanogaster* CREs (Figure 3C) [32]. These outcomes are consistent with evolutionary changes altering a *trans*-regulator of *tan* activation and *ebony* repression in *D. prostipennis*. However, the evolved *trans*-regulatory factor or factors currently remain unknown.

In another study, *tan* and *yellow* CREs from *D. auraria* were tested for their regulatory capability in transgenic *D. melanogaster*. While *yellow* and *tan* expression is limited to the A6 segment of this species, the CREs drove GFP expression in the A5 and A6 segments in a manner indistinguishable from that of *D. melanogaster* [21]. This outcome indicates that the regulatory logic with these two species yBE and t_MSE has not evolved, and imply modifications to the *trans*-regulatory landscape of this species. Moreover, in two independent cases in which male specific pigmentation was lost, it was found that the t_MSE for one species and the yBE for the other can still function in transgenic *D. melanogaster*. Thus, the lack of *yellow* and *tan* expression in these species likely results from changes to a *trans*-regulator. The importance of *trans*-regulatory evolution is further highlighted by two recent studies on convergent evolution, in which the underlying changes mapped in one case to the *pdm3* transcription factor gene [33], and in the other case to the

Abd-B transcription factor gene and *ebony* [34]. Collectively, these studies highlight how coordinated gene expression programs can often evolve by modifying *trans*-regulators that can affect only a subset of the co-expressed genes.

The role of pleiotropy in shaping the logical evolution of CREs

While much can be learned about trait evolution without knowing the specific transactions that mutations make with respect to the coming and going of transcription factor binding sites in CREs, this added depth of understanding allows one to probe increasingly complex phenomena, such as evolutionary constraint. In one recent study, it was found that *D. melanogaster* females with different extents of tergite pigmentation posses alleles of a CRE known as the "dimorphic element" that drives the pigment-repressing Bab transcription factor in the poster female abdomen [35]. These alleles differ in the number of segments and level of transcriptional activity, but share the ability to drive expression in the adjacently situated female genitalia (Figure 4A). The regulatory logic for this CRE possesses 14 binding sites for the Hox factor Abd-B, providing body-axis positional information, and two sites for Doublesex, the sex-specific regulator that restricts this CRE to the female abdomen and genitalia [36]. However, despite detailed knowledge of sixteen inputs to this CRE, the function-altering mutations of each allele occurred outside of these known binding sites, a trend which was also observed among other species at this CRE [35]. These outcomes indicate how the ancestral pleiotropic function of this CRE and its core regulatory logic could be maintained in the genitalia, while alterations occurred at other sites to modulate its more malleable abdominal function (Figure 4A). These observations demonstrate how knowledge of a CRE's logic can provide a mechanistic understanding of the constraints imposed on regulatory sequence evolution by pleiotropy.

A great degree of variation in male-specific pigmentation is seen for the A6 tergite of *D. auraria* males in Japan. For this intraspecific difference, *yellow* and *tan* expression similarly occurs in both highly pigmented and non-pigmented males. However, in the non-pigmented males, A6 expression of *ebony* is notably upregulated and this up-regulation is due to mutations that occurred in a CRE that functions as a transcriptional silencer element [37]. Thus, for this gene, the gain of expression occurred through mutational changes that attenuated the function of a silencer. It is possible that silencer inactivation provides a streamlined path to evolved increases in *ebony* expression (Figure 4B). Consistent with this scenario, the independent loss of pigmentation in *D. serrata* was similarly shown to result from increased *ebony* expression and the mutational modification of this species' silencer element [37]. Thus, the modification of a tissue-specific silencer, when present may be the easiest and least pleiotropic way to expand expression activities.

Conclusions

The results reviewed here show how obtaining a mechanistic view of CRE evolution and its relationship to phenotypes can reveal the molecular logic by which developmental systems change. We propose that the elucidation of how mutations affect a CRE's regulatory logic is a key to answering many of the looming questions in developmental evolution. Transcription factor binding events are the language by which the genome's instructions are read, and as

we now know, they play an important part in how those instructions change. Deciphering this language will allow us to understand why some passages change, while others cannot be edited. With the growing recognition that phenomena such as developmental plasticity, constraint, and robustness can play important evolutionary roles [38,39], traits like fruit fly pigmentation [40,41], for which the molecular basis of their construction and evolution have been worked out to the finest resolution will lead the way in providing concrete details of how these processes work mechanistically. Indeed, pigmentation of the female *D. melanogaster* posterior abdomen is influenced by temperature, and both *bab* [41] and *tan* [42] have been implicated in the environmental response. An understanding of these genes' CRE regulatory logic should allow insights to be made about the role of transcriptional regulatory sequences in the evolution of plasticity.

However, resolving regulatory logic presents a daunting challenge to the field. For example, the *D. melanogaster* genome has around 750 transcription factor genes [43] whose expression patterns are generally poorly characterized. Thus, connecting a single transcription factor to a functional binding event in a CRE remains laborious to establish [44]. In the face of these challenges though, the diversity of *Drosophila* pigmentation traits and their underlying CREs present an excellent model system to better understand transcriptional regulatory evolution, and are likely to lead the way into the mechanistic understanding regulatory logic evolution.

Acknowledgements

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Highlights

1. Modification of cis-regulatory elements (CREs) often underlie morphological evolution

2. Novel gene expression patterns can emerge via the modification of preexisting CREs

3. CREs with distinct transcription factor binding inputs can drive gene co-expression

4. Pleiotropic roles for transcription factor binding sites shape paths of CRE evolution

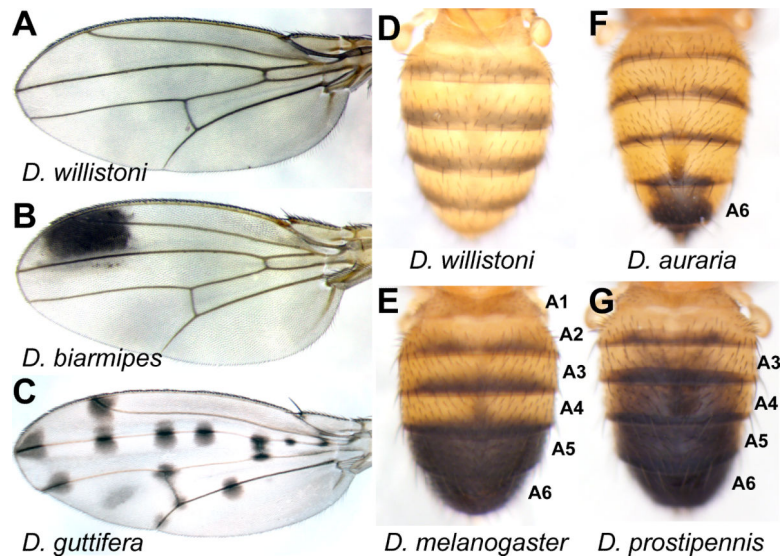5. Mechanistic grasp of CRE evolution will inform constraints, plasticity, canalization

Figure 1. Fruit fly pigmentation traits that serve as models for gene regulatory evolution
(A) The wings of *D. willistoni* are unpigmented. (B) *D. biarmipes* displays an antero-distal
black pigmentation spot. (C) The wings of *D. guttifera* flies are adorned with numerous
spots including those positioned where multiple veins connect, and at the positions of
campaniform sensilla. (D) Among *Drosophila* species, the dorsal surfaces of abdominal
segments 1 through 6 are covered by cuticle plates known as tergites that are typically only
partially pigmented, as shown for *D. willistoni*. (E) In *D. melanogaster* males, the A5 and
A6 segment tergites are fully pigmented. Male-specific pigmentation has (F) contracted to
the male A6 segment of *D. auraria* and (G) expanded to include the male A3-A6 segments of
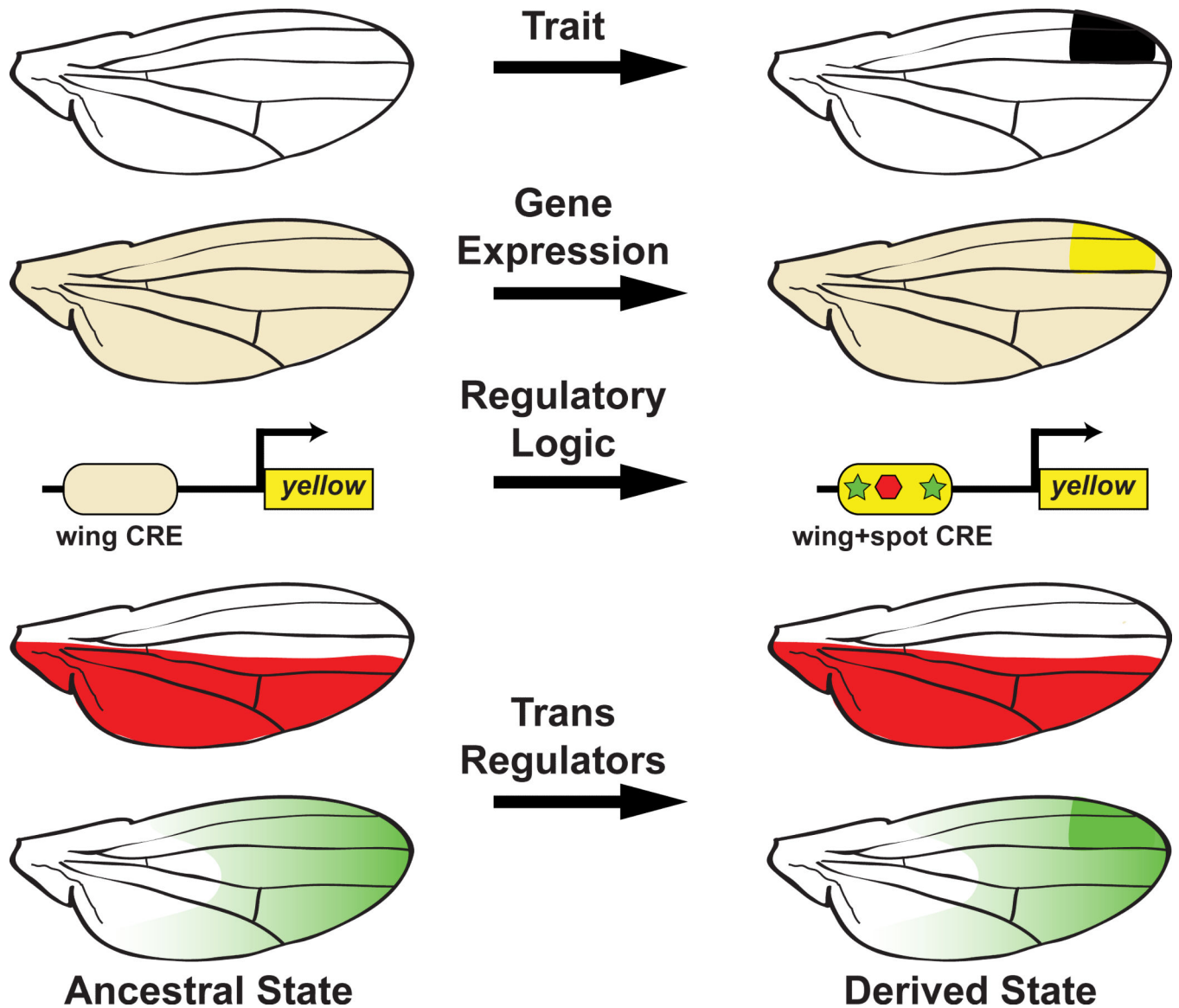*D. prostipennis*.

Figure 2. The origin of wing spots in *D. biarmipes* involved changes in *cis* and *trans* to the pigmentation-promoting gene *yellow*

A novel pattern of *yellow* gene expression accompanied the evolution of the *D. biarmipes* wing spot. The evolutionary path to this expression change involved converting a pre-existing wing CRE into the wing+spot CRE by evolving sites for Dll (green) as an activator and En (red) as a repressor. Along this evolutionary path, the Dll activator evolved a strong spot-prefiguring pattern of expression.
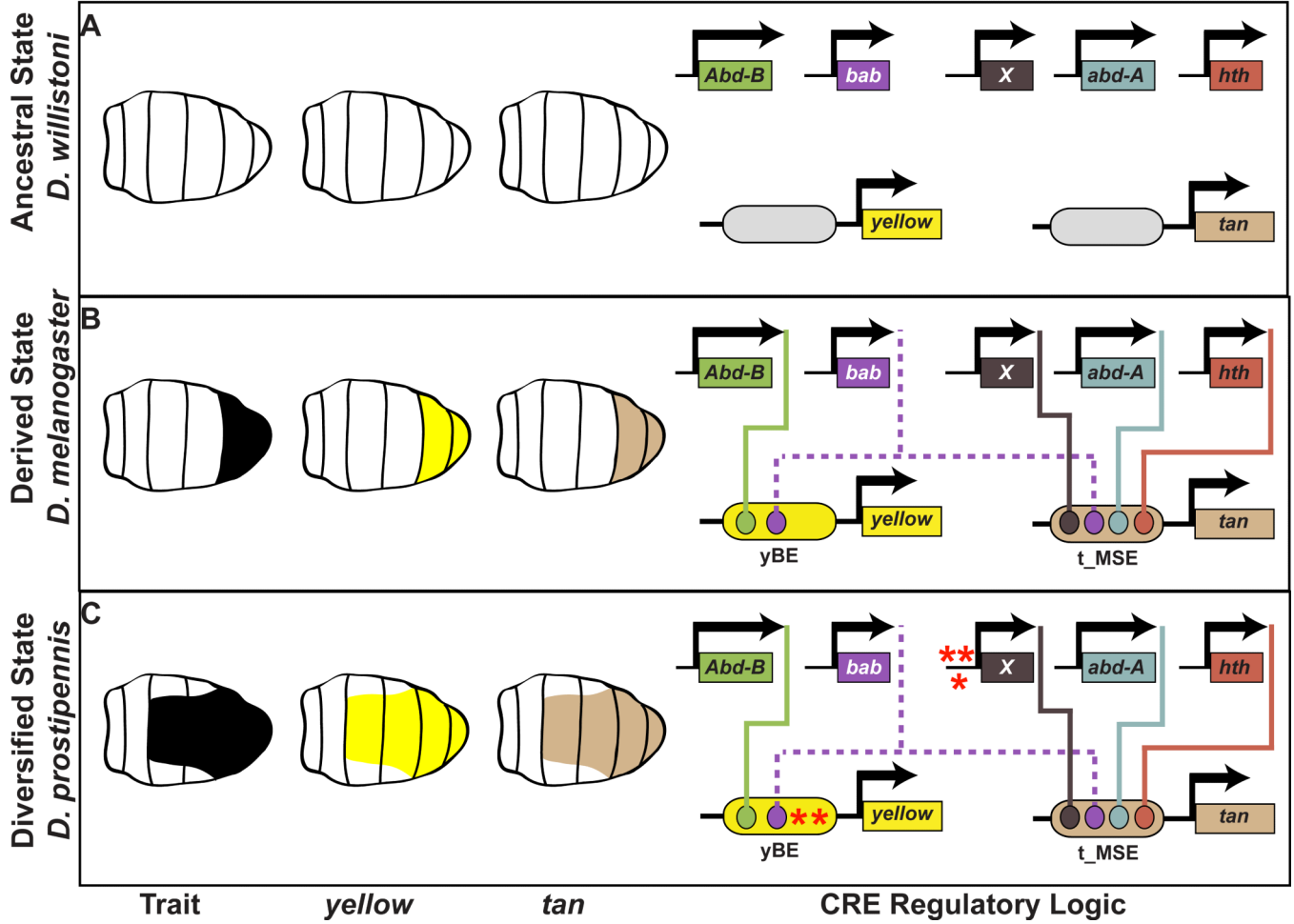
Figure 3. Mapping the origins and coordinated evolution of the *Drosophila* abdominal pigmentation network

(A) In the lineage of *D. melanogaster*, the ancestral state was inferred to have lacked male-specific tergite pigmentation in part because the *yellow* and *tan* genes were not expressed in the underlying male epidermis. The absence of *yellow* and *tan* expression corresponds with an absence of abdomen-specific CREs. (B) The derived male-specific tergite pigmentation originated through the gain of *yellow* and *tan* expression in the abdomen, driven by CREs containing binding sites for body plan-patterning transcription factors. (C) The expanded pigmentation observed on the abdomen of *D. prostipennis* males required coordinated expansion of *yellow* and *tan* gene expression. The maintenance of their co-expressed relationship during evolution involved modifications of the yBE, in the case of *yellow*, and alterations to an unknown *trans*-regulator(s), that altered the output of the t_MSE and thereby expression of *tan*. Red asterisks indicate the presence of function-modifying mutations.
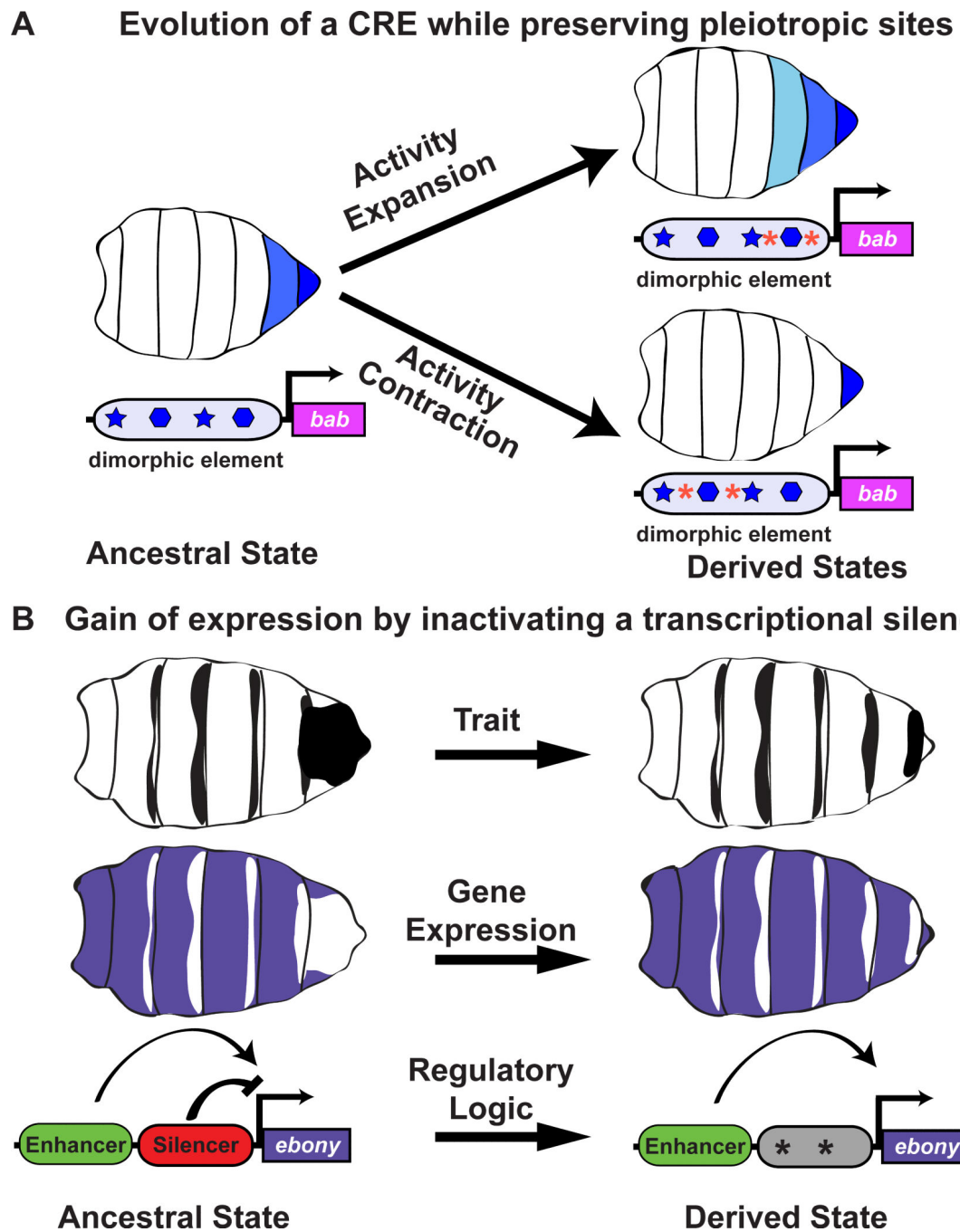
A   Evolution of a CRE while preserving pleiotropic sites

Ancestral State   **Derived States**

B   Gain of expression by inactivating a transcriptional silencer

Ancestral State   **Derived State**

Figure 4. Understanding CRE logic and architecture informs the nature of developmental constraints on evolution

(A) The dimorphic element is a CRE that activates the pigment repressor Bab in the female abdomen. Within *D. melanogaster* populations, the dimorphic element drives activity in a varying number of segments, ranging from many (light blue) to few (dark blue). Reconstruction of an ancestral enhancer for this species revealed that high and low activities evolved from an intermediate ancestor without disrupting 16 conserved binding sites (star and hexagon shapes) for two transcription factors. Hence, significant changes in abdominal activity evolved by function-altering mutations (red asterisks) that had no effect on the

binding sites that activate the enhancer in its ancestral genital setting (dark blue). (B) *D. auraria* males commonly have a dorsal pattern of pigmentation on the A6 tergite, which anti-correlates with the expression of the *ebony* (purple). Within Japan, some strains lack this pigmentation pattern, due to the gain of *ebony* expression throughout the A6 segment epidermis. This change in expression evolved through mutational changes (*) that abrogated the activity of a CRE that functions as a silencer element.