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Evolution of Developmental Control Mechanisms

The expansion of body coloration involves coordinated evolution in *cis* and *trans* within the pigmentation regulatory network of *Drosophila prostipennis*

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ABSTRACT

The generation of complex morphological features requires the precisely orchestrated expression of numerous genes during development. While several traits have been resolved to evolutionary changes within a single gene, the evolutionary path by which genes derive co-localized or mutually excluded expression patterns is currently a mystery. Here we investigate how the *Drosophila* pigmentation gene network was altered in *Drosophila prostipennis*, a species in the *Drosophila melanogaster* subgroup, that evolved expanded abdominal pigmentation. We show that this expansion involved broadened expression of the melanin-promoting enzyme genes *tan* and *yellow*, and a reciprocal withdrawn pattern of the melanin-suppressing enzyme gene *ebony*. To examine whether these coordinated changes to the network were generated through mutations in the *cis*-regulatory elements (CREs) of these genes, we cloned and tested CREs of *D. prostipennis tan*, *ebony*, and *yellow* in transgenic reporter assays. Regulatory regions of both *tan* and *ebony* failed to recapitulate the derived *D. prostipennis* expression phenotype, implicating the modification of a factor or factors upstream of both genes. However, the *D. prostipennis yellow cis*-regulatory region recapitulated the expanded expression pattern observed in this species, implicating causative mutations in *cis* to *yellow*. Our results provide an example in which a coordinated expression program evolved through independent changes at multiple loci, rather than through changes to a single "master regulator" directing a suite of downstream target genes. This implies a complex network structure in which each gene may be subject to a unique set of inputs, and resultantly may require individualized evolutionary paths to yield correlated gene expression patterns.

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Introduction

An immense degree of orchestration directs the production of fully formed adults from the single-celled zygote. During the development of any tissue or cell type, dozens if not hundreds of genes must be activated to establish the presence of proteins that work together to produce the tissue's physical properties. Such gene expression programs are coordinated by gene regulatory networks (GRNs) composed of transcription factors and signaling pathways that direct the timing and spatial patterning of each participating gene (Davidson, 2001). Genes regulated by the network are activated by their *cis*-regulatory elements (CREs), short stretches of DNA containing individual binding sites for factors of the network that, combined, determine timing and spatial distribution of the gene product (Small et al., 1992). Often, mutually exclusive patterns of gene expression are established – activating one gene in a zone complementary to others. Such reciprocal patterns of expression are commonly observed in signaling pathways and negative feedback loops, as the participants frequently play an active role in repressing their mutually excluded targets (Boisclair Lachance et al., 2014; Heitzler et al., 1996; Müller et al., 2003). While correlated and reciprocal patterns of expression are general features of developmental networks, it remains an open question how such mutually exclusive configurations form and change during evolution.

The rapidly evolving pigmentation patterns of *Drosophila* species have served as a useful model for elucidating the nature of developmental evolution (Gompel et al., 2005; Jeong et al., 2008; Kopp et al., 2000; Rogers et al., 2014; True et al., 1999; Williams et al., 2008; Wittkopp et al., 2002b). Pioneering genetic studies of the model organism *Drosophila* (*D.*) *melanogaster* mapped the genes and

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enzymatic pathways that generate pigmentation patterns (reviewed in Wittkopp et al., 2003). In addition, a great diversity of pigmentation phenotypes exist in species closely related to *D. melanogaster* (Gompel and Carroll, 2003; Kopp and True, 2002; Kopp et al., 2000; Lachaise et al., 2000; Prud'homme et al., 2007; Salomone et al., 2013), allowing the employment of a rich set of molecular and genetic tools of this premiere model organism to investigate the evolution of these phenotypes.

Studies into the elegantly pigmented wings of *Drosophila* species in the oriental lineage have provided several examples of mutually exclusive gene expression patterns contributing to phenotypic evolution (Gompel et al., 2005; Wittkopp et al., 2002a). The darkly colored melanic wing spot of *Drosophila biarmipes* correlates with strong expression of the pigment-promoting enzyme Yellow and the coincident down-regulated expression of Ebony in the territory that forms the spot (Wittkopp et al., 2002a). A detailed analysis of the *D. biarmipes* wing regulatory landscape revealed that expression of the transcription factor Distalless had evolved to presage the spot pattern (Arnoult et al., 2013). In a series of experiments that manipulated *D. biarmipes* Distalless expression, Arnoult et al. discovered that Distalless is required for both activation of *yellow* and repression of *ebony*. Their finding that divergent spot morphologies tend to correlate with divergent Distalless expression suggested that once connections between Distalless and the pigmentation genes were formed, the simplest path to generate coordinated changes in the network was through the alteration of Distalless expression (Arnoult et al., 2013). However, in *D. biarmipes* and other cases of spotted species, the molecular basis of how complimentary "anti-spots" of *ebony* evolved has not been identified as the relevant CREs have not been mapped. Investigating this complex relationship in a tissue in which the CREs of *ebony* are well characterized, would offer new opportunities to study the evolutionary diversification of mutually exclusive patterns of *ebony* and *yellow*.

Recently, the reciprocal relationship between pigment promoting and repressing enzymes was extended to the dimorphically pigmented cuticular plates (tergites) of *Drosophila melanogaster* and its close relatives (Jeong et al., 2008; Rebeiz et al., 2009a; see Fig. S1A). Expression of *yellow* is localized to zones of dark pigment, including the two posterior-most tergites in males (Walter et al., 1991; Wittkopp et al., 2002a). The *tan* gene, that similarly promotes dark pigmentation (True et al., 2005), is co-expressed with *yellow* in these body segments (Jeong et al., 2008). Abdominal expression of *ebony* was found to inversely correlate with black melanin patterns (Rebeiz et al., 2009a), with expression restricted to the anterior unpigmented body segments of the male. Within *ebony*-expressing segments, *ebony* mRNA is restricted from the posterior edge of each tergite, where darker pigments form.

While a single positive-acting CRE controls *yellow* (Jeong et al., 2006; Wittkopp et al., 2002b) and *tan* (Jeong et al., 2008), *ebony* expression is controlled by three CREs (Rebeiz et al., 2009a). A CRE located 2.5 kb upstream of the *ebony* promoter activates reporter gene expression throughout the abdomen, including the previously mentioned darkly pigmented regions (Rebeiz et al., 2009a). Two additional CREs function as silencers of the activator element. One silencer lies adjacent to the *ebony* promoter and functions to restrict expression from pigmented body segments of the male (the "male repression element"). The second silencer resides in the first intron (the "stripe repression element") and functions to restrict *ebony* expression from the posterior edges of tergites that form pigmented regions. These silencers inactivate *ebony* expression in zones in which *yellow* and *tan* expression occurs. This raises the question of how these elements participate in the evolution and divergence of these reciprocal patterns. Are correlated and mutually exclusive patterns of *yellow*, *tan*, and *ebony* maintained during evolution through the independent alteration of their CREs (Fig. S1B)? Or must a "master regulator" (*e.g.* Distalless in the *D. biarmipes* wing spot) exist that can control activation and repression, such that changes to this single factor are sufficient to orchestrate concerted evolution of the pigmentation program (Fig. S1C)?

Here, we investigate the coordinated evolution of the pigmentation gene network underlying a recent drastic change in male coloration phenotype. In a broad screen of pigmentation phenotypes in the *Drosophila melanogaster* species group, we identified a pronounced expansion of the male specific phenotype of *Drosophila prostipennis*, a close relative of *D. melanogaster*. Examining the state of pigmentation gene expression, we found that the phenotypic changes in *D. prostipennis* are accompanied by coordinated changes in *yellow*, *tan*, and *ebony* expression. To examine whether these synchronized differences are due to changes in the CREs for these genes, we tested the activity of regulatory elements of *tan*, *ebony*, and *yellow* in reporter assays carried out in transgenic *D. melanogaster*. We found that a combination of direct changes to the regulatory element of *yellow*, as well as alterations in *trans* to *ebony* and *tan* contributed to this phenotype. These results imply that the evolution of complimentary changes in gene expression can evolve independently, suggesting that existing regulatory landscapes are sufficiently complex to evolve coordinated expression patterns in unique ways.

Materials and methods

Fly strains and husbandry

Stocks were maintained at room temperature on standard media. Most stocks of species used in this study (Table S1) were obtained from the UCSD *Drosophila* Species Stock Center (http://stockcenter.ucsd.edu).

In situ hybridizations

In situ hybridization was performed as previously described (Jeong et al., 2008). In brief, pupal samples were aged to differing extents for each probe (85–90 h after pupal formation (hAPF) for *yellow*, 90–95 hAPF for *tan*, and at eclosion for *ebony*), dissected in cold PBS, and fixed in 4% paraformaldehyde (E.M.S. Scientific). PCR was performed to generate DNA templates to be used in transcription of antisense RNA probes. Transcription was initiated from a T7 promoter appended *via* primer design. See Table S2 for probe primers used in this study. Digoxigenin-labeled probes were generated using a 10 × Dig labeling mix (Roche Diagnostics) and T7 RNA polymerase (Promega). Because *D. prostipennis* and *Drosophila takahashii* are very closely related (90–95% nucleotide sequence identity based on alignments of *D. prostipennis* sequences to the *D. takahashii* genome), spatial differences in pigmentation gene expression could reliably be detected with the same probe for both species.

GFP reporter assays

CREs were combined with a heterologous promoter and a coding sequence for a nuclear localized Green Fluorescent Protein (GFP) as previously described (Rebeiz et al., 2009a). Using the GenePalette Software tool (Rebeiz and Posakony, 2004), primers were designed to target conserved sequences and used to amplify *D. prostipennis tan* and *ebony* CREs. Restriction sites (*Asc I* and *Sbf I*) were appended to primers (Integrated DNA Technologies), and PCR products were cloned into the S3aG vector (Williams et al., 2008), which contains *SfI* and *gypsy* insulators flanking the expression cassette. Insertion of the *ebony* intron 3′ to GFP was performed by Infusion cloning

(Clontech) into a *Spe I* site. See Table S3 for primers used to clone regulatory constructs in this study. Transformant lines were generated by phi-C-31 mediated site specific recombination into the 51D insertion site (Bischof et al., 2007). Transgenic *D. melanogaster* was mounted on slides in halocarbon oil and imaged on an Olympus Fluoview 1000 confocal microscope. For *tan* and *yellow* reporter constructs, pupae were imaged during the last day of pupal development ~85 to 95 hAPF. For *ebony* reporters, samples were aged for 24 h after eclosion before imaging.

Results

A survey of Drosophila pigmentation phenotypes

In order to identify recent shifts in pigmentation, we performed a broad phenotypic screen of the *melanogaster* species group (O'Grady and Kidwell, 2002). The *Drosophila* abdomen is rigidly constrained in the number and shape of body segments. *Drosophila* males have six abdominal segments ("A1"–"A6") that form tergites, while females bear an additional A7 body segment that is reduced and triangular in shape (Fig. 1). Referencing the phylogeny of Jeong et al. (2006), species of the oriental and *montium* lineages (Fig. S2) were compared to selected species from the *ananassae* and *obscura* groups.

Within the *D. melanogaster* subgroup, a clade of 9 species which contains *D. melanogaster*, the most drastic shift in pigmentation was the complete loss of sexually dimorphic melanic coloration exhibited by *Drosophila santomea* (Lachaise et al., 2000) (Fig. 1). Overall, however, we observe very few changes in male-specific tergite pigmentation. A major shift occurs in the *montium* lineage, the sister clade to the oriental lineage. In this large group of species, the extent of pigmentation is reduced from two fully pigmented tergites to one (Fig. S2). Further, several species in the *montium* clade have lost male-specific pigmentation. Two major clade-wide losses of pigmentation seem to have occurred within this lineage, specifically those containing *Drosophila birchii* and *Drosophila kikkawaii*.

Additional exceptions to the invariance of male-specific pigmentation phenotypes exist within the oriental lineage. In the *elegans* subgroup, we note an expansion of male-specific pigmentation in *Drosophila lucipennis* (Fig. 1). *Drosophila eugracilis*, which is basal to the *melanogaster* subgroup, shows a similar expansion of pigmentation into A4, and part of the A3 tergite. Within the *takahashii* subgroup, we observed a drastic expansion of pigmentation in the species *D. prostipennis* (Figs. 1 and 2A and B). In all of these cases, pigmentation expanded beyond the A5 and A6 body segments that typify this group, into more anterior A4 and A3 segments. We sought to characterize the molecular underpinnings of the expanded pigmentation of *D. prostipennis*.

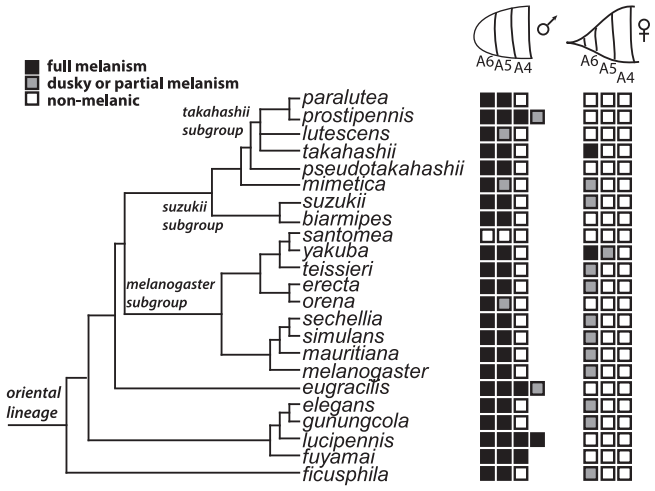

Fig. 1. Evolutionary shifts in pigmentation phenotypes among species of the oriental *Drosophila* lineage. Species from Table S1 were surveyed for pigmentation phenotypes in the A6–A4 tergites of males (left) and females (right). Fully melanic tergites are denoted by black squares, while pigmentation that formed an incomplete tergite pattern, or was not a dark black color is indicated by a gray square. Unpigmented segments are denoted by a white square. The phylogeny is based upon that of Jeong et al. (2006).

Coordinated alteration of the pigmentation enzyme network accompanies expanded pigmentation

As several previously characterized studies of abdominal pigmentation involve the loss or reduction of pigment (Jeong et al., 2008, 2006; Rebeiz et al., 2009a; Rogers et al., 2013), the phenotype of *D. prostipennis* represented a rare opportunity to study a recent spatial expansion of pigmentation. Previous studies in the *D. melanogaster* subgroup had shown that the *yellow* and *tan* genes are expressed in a sex-specific manner throughout posterior tergites that are fully pigmented in males (Jeong et al., 2008; Wittkopp et al., 2002a). A recent study of the *D. melanogaster ebony* gene revealed a reciprocal pattern of expression, displaying *ebony* transcripts only in anterior regions of segments underlying the unpigmented zone of the male A2–A4 tergites (Fig. S1), regions that lack *yellow* and *tan* expression (Rebeiz et al., 2009a). To assess the extent to which this network was modified in *D. prostipennis*, we employed *in situ* hybridization to compare the spatial deployment of these three genes in *D. prostipennis* to those of *D. takahashii*, a closely-related species that has the ancestral *melanogaster*-like pattern of male-specific pigmentation (Fig. 2A).

The strongest expression of *yellow*, *tan*, and *ebony* occurs at distinct stages of abdominal development. The *yellow* gene is expressed during the third day of pupal development between 60 and 85 hAPF (Jeong et al., 2008; Wittkopp et al., 2002a). Performing *in situ* hybridizations for *yellow* at this stage revealed that *D. takahashii*, much like *D. melanogaster*, has strong levels of *yellow* expression throughout the A5 and A6 tergites of male pupae (Fig. 2C). In contrast, *D. prostipennis* males show an expansion of *yellow* expression that extends into the more anterior A3 and A4 segments (Fig. 2D). The expression of *yellow* in this territory correlates with the spatial distribution of dark pigment in *D. prostipennis* (Fig. 2D′, arrows).

In a trend similar to that detected for *yellow*, we observed a strong correlation between *tan* expression and the *D. prostipennis* phenotype. The optimal stage of *tan* expression is delayed relative to *yellow*, with transcript significantly accumulating *circa* ~90 hAPF in a pattern that is maintained through eclosion of the fly (Jeong et al., 2008; Rebeiz et al., 2009b). *D. takahashii* males exhibit strong, tergite-wide expression of *tan* in the darkly pigmented A5 and A6 body segments (Fig. 2E), with no expression in the A3 or A4 segments (Fig. 2E, arrows). In *D. prostipennis*, this zone is expanded, with near-complete expression throughout the center of the A4 tergite and slight expression at the posterior edge of the A3 tergite (Fig. 2F, F′, arrows). Considering our results with *yellow* and *tan*, the anterior expansion of pigmentation in *D. prostipennis* is accompanied by the expanded expression of these two enzymes.

To test whether these expanded domains of *yellow* and *tan* expression were coordinated with reciprocal changes reducing the spatial distribution of *ebony*, we examined its deployment in *D. prostipennis* and *D. takahashii*. Although a broad, low-level of Ebony protein has been detected during pupal development (Wittkopp et al., 2002a), its peak patterned expression occurs just after eclosion of the adult fly (Rebeiz et al., 2009a). In *D. takahashii*, we observe the expected *melanogaster*-like pattern of *ebony*, with

high levels of transcript accumulating anteriorly (A2–A4), and little signal detected in the highly pigmented A5 and A6 tergites (Fig. 2G). In A2–A4 of *D. takahashii*, *ebony* transcript accumulates uniformly throughout the anterior three quarters of the tergite, correlating well with the pattern of dark stripes that form on each abdominal segment (Fig. 2A). *D. prostipennis* males also show a similar lack of expression in the A5 and A6 segments (Fig. 2H). However, in the male A3 and A4 tergites, *ebony* is restricted to lateral portions of the tergite, with little or no expression in the dorsal midline region (Fig. 2H, bracket). This pattern of *ebony* expression correlates with the pattern of yellow colored cuticle in these segments (Fig. 2B, bracket).

These results suggest the changes in abdominal pigmentation that evolved in *D. prostipennis* occurred in a coordinated fashion such that these three pigmentation enzymes preserved co-expressed as well as mutually exclusive patterns. As the steps involved in such coordinated evolution of gene expression programs are poorly understood, we next sought to determine the mechanism by which this gene expression program evolved.

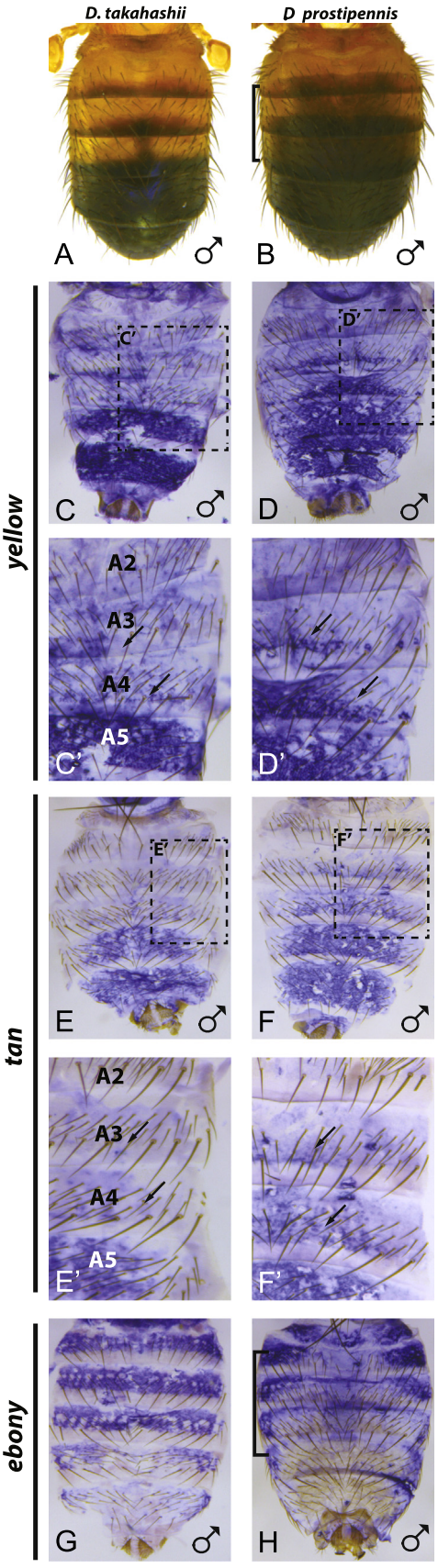

Fig. 2. Coordinated changes in pigmentation gene expression correlate with the derived phenotype of *D. prostipennis*. (A and B) Abdominal pigmentation phenotypes of *D. takahashii* (A) and *D. prostipennis* (B) males. The bracket in (B) highlights the expanded pigment into the A3 and A4 tergites. (C–D′) Abdominal *yellow* mRNA in *D. takahashii* (C and C′) and *D. prostipennis* (D and D′) males shown by *in situ* hybridization in 75–85 hAPF pupae. The dashed boxes in (C) and (D) are enlarged in (C′) and (D′) to show the expansion of the *yellow* transcript into the A4 and A3 tergites in *D. prostipennis* (arrows). (E–F′) Abdominal *tan* mRNA expression in 85–95 hAPF pupal samples of *D. takahashii* (E and E′) and *D. prostipennis* (F and F′). The dashed box in (F) is enlarged to detail the expansion of *tan* transcript into A4 and the posterior most section of A3 ((F′) compare with (E′) where there is no expansion of *tan* transcript). (G and H) *ebony* transcript visualized in newly enclosed flies. The bracket in (H) highlights where *ebony* expression is limited to the most lateral edges of the A4 and A3 tergites (compare to (G)). Images of *D. prostipennis* and *D. takahashii* female expression patterns are shown in Fig. S3.

Changes in trans to tan and ebony caused coordinated shifts in expression

Several evolutionary scenarios could explain the patterns of correlated and mutually exclusive expression that we observe in *D. prostipennis* (Fig. S1). First, the coordinated changes could be due to independent modifications of the *tan*, *yellow*, and *ebony* CREs themselves (Fig. S1B). Alternately, these divergent patterns of expression could arise through the alteration of one or more upstream regulatory factors that regulate this trio of downstream target genes (Fig. S1C). Finally, it is possible that a combination of these mechanisms could account for the transitions in expression that took place. Considering these possible mechanisms, the number of genes that must change to generate these diverse patterns of expression differs greatly. If each gene were modified individually, all three genes would accumulate changes to generate the concerted program of development. On the other hand, changes to a single *trans* regulatory factor could potentially alter all three genes' expression through direct or indirect downstream connections with CREs. Finally, if even one of these three enzyme genes was modified by *cis* regulatory changes, it would suggest that multiple genes must have been altered during the evolution of this coordinated program of gene expression. To distinguish between these hypotheses, we tested the regulatory regions of *D. prostipennis tan*, *ebony*, and *yellow* in a GFP reporter assay. These assays were performed in the common genetic background of *D. melanogaster*, which exhibits the ancestral patterns of both pigmentation and gene expression for this clade (Fig. 1). Due to our insertion of transgenes into the same site in the genome (Bischof et al., 2007), this assay is able to detect slight differences in gene regulatory activity that can be attributed to CRE evolution.

The abdominal expression of *tan* is controlled by the male specific element (t_MSE), a CRE located upstream of the promoter (Fig. 3A). It is situated between two upstream genes and is required for the rescue of abdominal pigmentation phenotypes

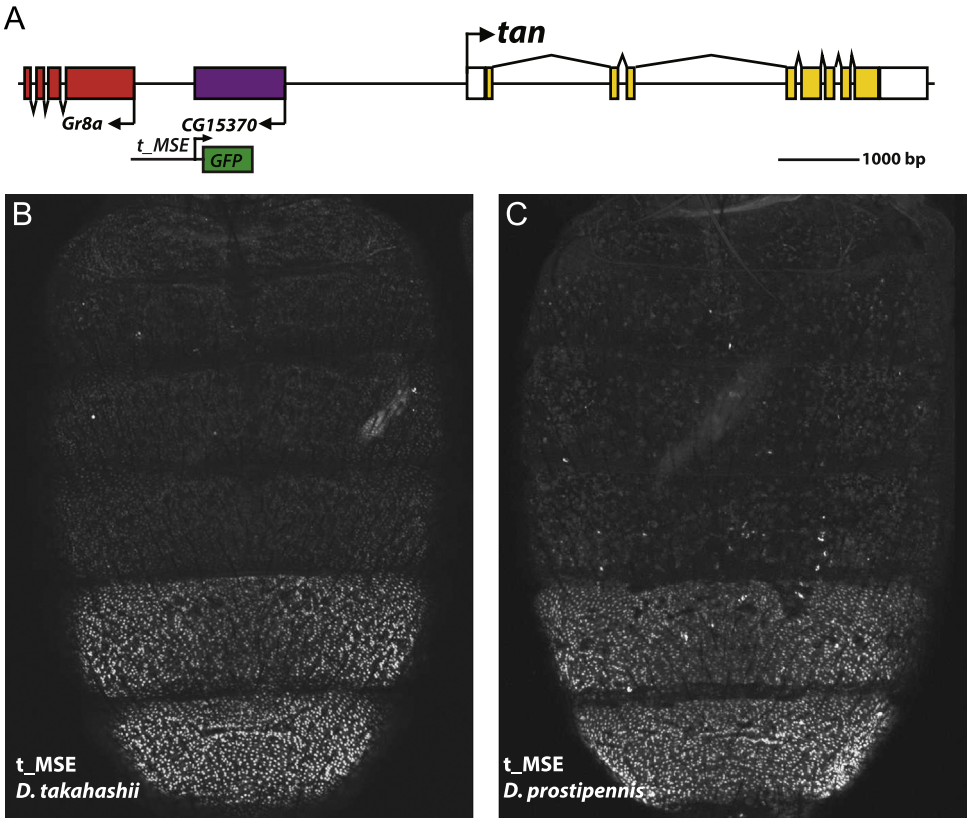

Fig. 3. Activity of the *D. prostipennis tan* CRE in transgenic *D. melanogaster*. (A) Map of the *tan* locus, the *tan* male specific element ("t_MSE") indicates the CRE region driving the expression of a GFP reporter. (B and C) Activity of t_MSE reporter transgenes of *D. takahashii* (B) and *D. prostipennis* (C) in male late pupal abdomens (~90 to 96 hAPF). Images of female pupal abdomens are shown in Fig. S3.

of *tan* in another lineage (Jeong et al., 2008). The orthologous *D. takahashii* t_MSE generates sex-specific expression of *tan* throughout the male A5 and A6 tergites, recapitulating the endogenous pattern of *tan* expression in *D. takahashii* (Fig. 3B). This indicates that the activity of the t_MSE is conserved between the *takahashii* and *D. melanogaster* subgroups. To directly compare the activity of the *D. prostipennis* t_MSE, we cloned this sequence from the *D. prostipennis* genome, and generated transgenic reporter lines in the same background as the *D. takahashii* construct. If the expanded expression pattern of *tan* is due to *cis*-regulatory changes in this CRE, we would expect that the *D. prostipennis* t_MSE would drive expanded GFP expression limited to the medial regions of the A4 and A3 male segments. However, the *D. prostipennis* t_MSE construct drove expression of GFP in a pattern identical to the *D. takahashii* orthologous CRE (Fig. 3C). To confirm that a possible expansion of *D. prostipennis* CRE activity is not temporally separated from the A5–A6 activity, we tested several timepoints from early pupae through eclosion; however we failed to find an expansion in GFP activation that recapitulates the *D. prostipennis* pattern of *tan* expression (not shown). These results suggest that the expanded expression of *tan* in *D. prostipennis* is due to changes in factors upstream of this conserved CRE.

The sexually dimorphic expression of *ebony* is controlled by two CREs upstream of the promoter (Rebeiz et al., 2009a). The upstream abdominal enhancer drives activation throughout the abdomen (Fig. 4A, "abd"). The activity of this enhancer is antagonized in the male A5 and A6 segments by a promoter proximal silencer element (Fig. 4A, "male rep"). When the full upstream region of *D. melanogaster ebony* (Fig. 4A, "*ebony* upstream reporter") is assayed in a GFP reporter construct, strong expression is observed in the A1–A4 body segments, with only bristle expression visible in A5 and A6 segments (Fig. 4B). Using primers that correspond to conserved sequences, we fused the full 7 kb upstream region of *D. prostipennis ebony* into a GFP reporter construct, which was inserted into the same genomic position as the *D. melanogaster ebony* construct. Similar to *D. melanogaster*, the *D. prostipennis ebony* reporter drove robust expression in anterior body segments A1–A4 (Fig. 4C), and was absent throughout the A5 and A6 segments. Hence, we can conclude that the activity of the *D. prostipennis* male repression element is conserved and that *trans* rather that *cis*-regulatory evolution is responsible for the evolved pattern of *D. prostipennis ebony* expression.

To address the possibility that the receding pattern of *ebony* expression in *D. prostipennis* is due to changes in the intronic "stripe repression" element, we also tested constructs that included both upstream and intronic sequences (Fig. 4A, "*ebony* upstream+intron reporter"). The *D. melanogaster* reporter was excluded from the A5 and A6 body segments, but also showed strong repression at the posterior edges of each tergite (Fig. 4D, inset). Similarly, the *D. prostipennis* upstream+intron reporter exhibited enhanced repression at the posterior edges of tergites, but still failed to recapitulate the withdrawn spatial expression of *ebony* observed in this species (Fig. 4E, compare inset to that of Fig. 4C). From this data, we conclude that the drastic shift from the *D. melanogaster* to the *D. prostipennis* pattern was largely caused by changes in *trans* to *ebony*.

cis-regulatory changes in yellow contributed to its expanded pattern of expression in D. prostipennis

To examine whether the expanded expression of *yellow* was due to the alteration of its regulatory sequences, we cloned the region responsible for body expression (Fig. 5A) from *D. takahashii* and *D. prostipennis* into our reporter system. The study by Jeong et al., had found that a 2.6 kb region containing the previously identified

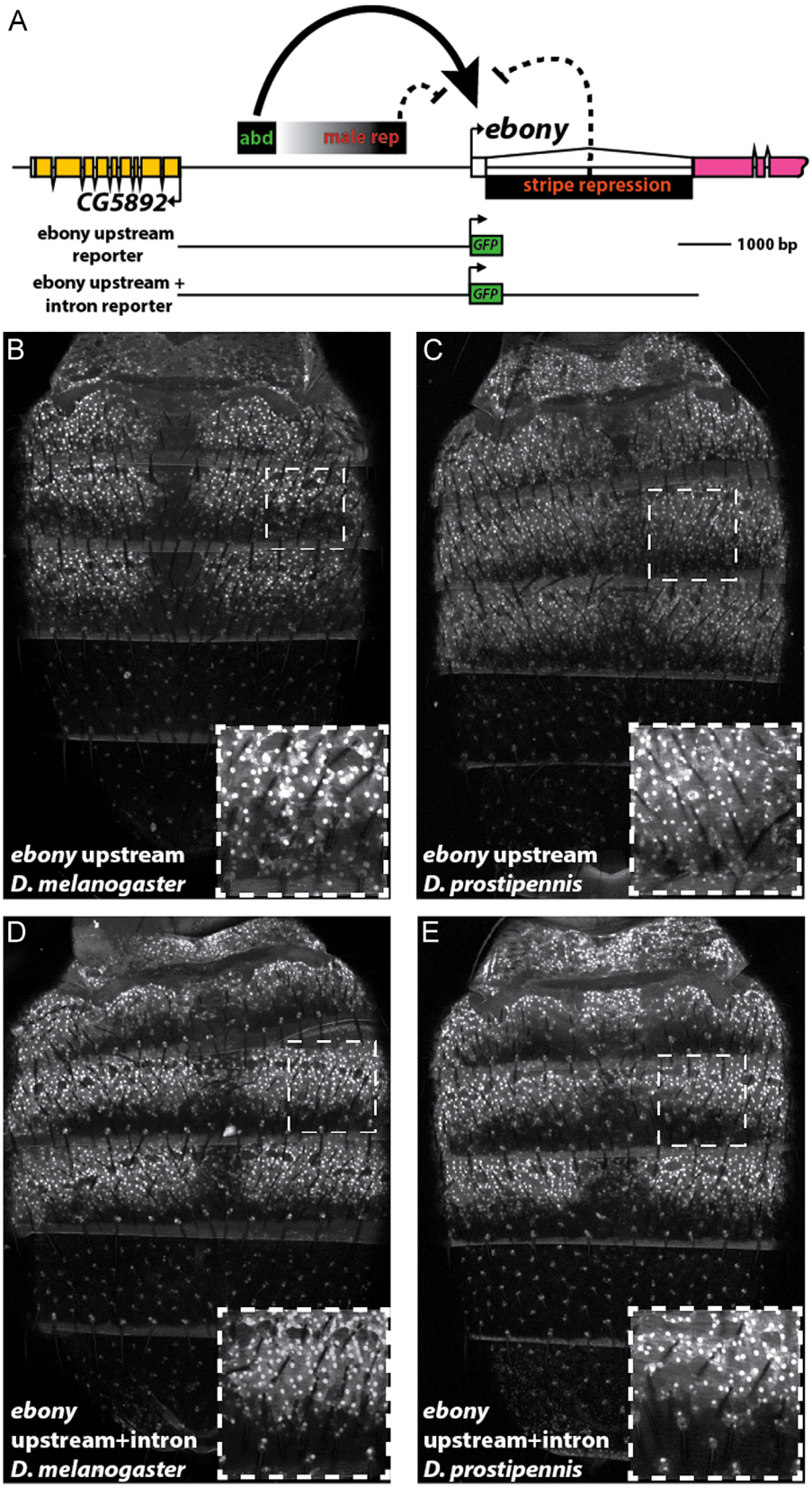

Fig. 4. Activity of the *D. prostipennis ebony* regulatory region in a *D. melanogaster* background. (A) Map of *ebony* locus displaying the positive-acting abdominal enhancer ("abd") and two silencer regions: "male rep," which restricts expression from male A5 and A6 segments, and the intronic "stripe repression" element that represses activation at the posterior edges of tergites. Schematics of GFP reporter constructs that include upstream and intronic regions. (B and C) The *ebony* upstream reporter of *D. melanogaster* (B) and *D. prostipennis* (C) males. (D and E) The *ebony* upstream+intron construct of *D. melanogaster* (D) and *D. prostipennis* (E) males. Images of female abdomens are shown in Fig. S3. *ebony* reporters are best imaged several hours post-eclosion. At this time point, however, images of the full upstream region appear to have decreased expression at the posterior edge of tergites due to the formation of pigments that interfere with imaging. Insets displaying the region outlined with a dashed line provide a close-up view in which stripe repression activity can be visualized.

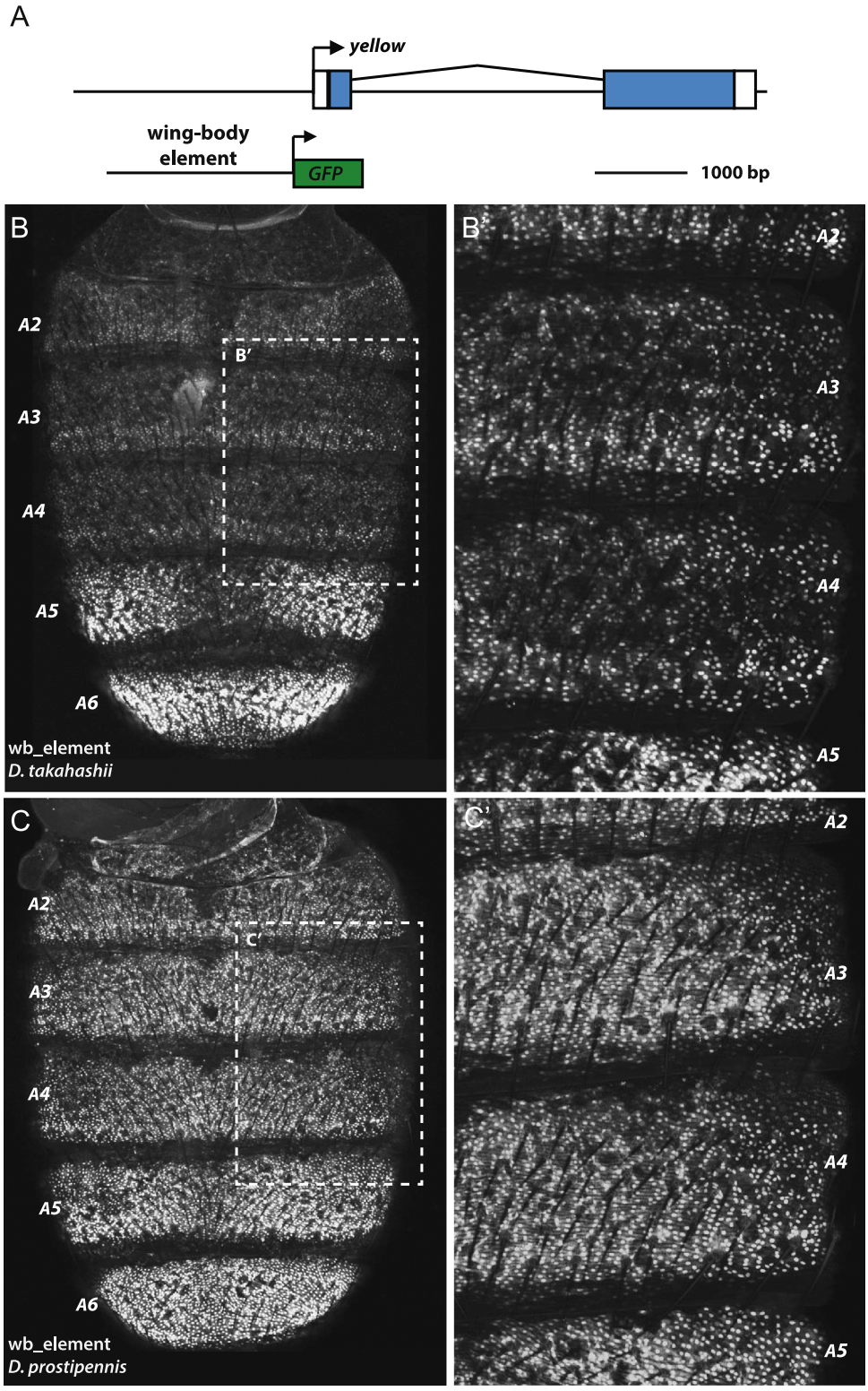

Fig. 5. Activity of the *D. prostipennis yellow* regulatory region. (A) Map of the *yellow* gene locus, "wb_element" indicates the upstream regulatory region used to drive the reporter construct shown in *D. takahashii* (B and B′) and *D. prostipennis* (C and C′) at 85 hAPF. (B′–C′) The dashed box region in (B) and (C) is enlarged to show upregulated GFP in A4–A3 of *D. prostipennis* construct (C′) when compared to *D. takahashii* (B′). Images of female pupal abdomens are shown in Fig. S3.

"wing" and "body" regulatory sequences (wb_element) (Geyer and Corces, 1987; Wittkopp et al., 2002b) of *yellow* was required to fully recapitulate abdominal *yellow* expression in a reporter assay (Jeong et al., 2006). Further, several studies have established that this CRE originated prior to the divergence of the species studied here (Jeong et al., 2006; Kalay and Wittkopp, 2010; Wittkopp et al., 2002b).

Testing the wb_element reporter of *D. takahashii*, we observed strong expression in the A5 and A6 body segments, recapitulating the *D. takahashii yellow* expression pattern (Fig. 5B). In contrast, the *D. prostipennis* wb_element reporter drove GFP expression in an expanded pattern resembling the *D. prostipennis* pigmentation pattern (Fig. 5C). Notably, the expression driven by this reporter in the A4

tergite strongly recapitulates the endogenous dorsally upregulated expression that is observed in *D. prostipennis* (compare Fig. 5C′ to Fig. 2D′). In more anterior A2 and A3 body segments, expression of the reporter was also expanded, although the extent of expansion is broader than the endogenous expression of *D. prostipennis yellow* in these segments. Thus, from this data, we conclude that the expanded expression of *yellow* in *D. prostipennis* resulted in large part from changes to its *cis*-regulatory region.

Discussion

We have shown how the coordinated evolution of a gene expression program occurred through heterogeneous changes in both *cis*-regulatory elements of a terminal pigmentation enzyme and the *trans*-regulatory landscape of factors that pattern these enzymes in the *Drosophila* abdomen (Fig. 6). In a large survey of abdominal coloration phenotypes across the *melanogaster* species group, we identified several recent transitions in phenotype that included gains and expansions of pigmentation. Dissecting an individual instance of pigment expansion, we found that three genes, *yellow*, *tan*, and *ebony*, have derived coincident patterns of expanded and contracted expression (Fig. 6A). Our functional tests of *tan* and *ebony* CREs revealed that the drastic and concerted expression changes in these genes arose without directly altering their regulatory sequences, strongly implicating modifications of one or more upstream factors that regulate these two enzymes (Fig. 6B, bottom). However, we uncovered evidence that the *yellow* gene incorporated *cis*-regulatory mutations that contributed to its expanded pattern of expression (Fig. 6B, top). Hence, the *trans*-regulatory changes affecting *tan* and *ebony* did not necessarily extend to *yellow*. These results suggest that the regulatory complexity of the pigmentation network *trans*-landscape is complex to the point where the re-patterning of expression for multiple genes readily proceeds through individualized paths of *cis*- and *trans*-evolution.

The Drosophila abdomen as a model for the co-evolution of reciprocal expression programs

The reciprocal expression of *ebony* and *yellow* was first reported in the single wing spot of *D. biarmipes* (Wittkopp et al., 2002a) and was subsequently extended to the complex pattern of spots observed in *Drosophila guttifera* (Gompel et al., 2005), suggesting that mutually exclusive patterns of these genes arise frequently and may be required for melanic pigmentation. Recent studies have shown how this trend of mutually exclusive expression extends to the highly varied patterns of abdominal pigmentation in *Drosophila* (Jeong et al., 2008; Rebeiz et al., 2009a). Moreover, the regulatory sequences that mediate the respective patterns of these genes are known for the abdomen, which provides a unique opportunity to study the co-evolution of this gene expression program. Here, we expanded upon previous observations to show that *tan*, encoding the enzyme that performs the reverse reaction of *ebony* (True et al., 2005), collaborates with *yellow* and *ebony* in the coordinated evolution of a derived abdominal pigmentation pattern (Fig. 2).

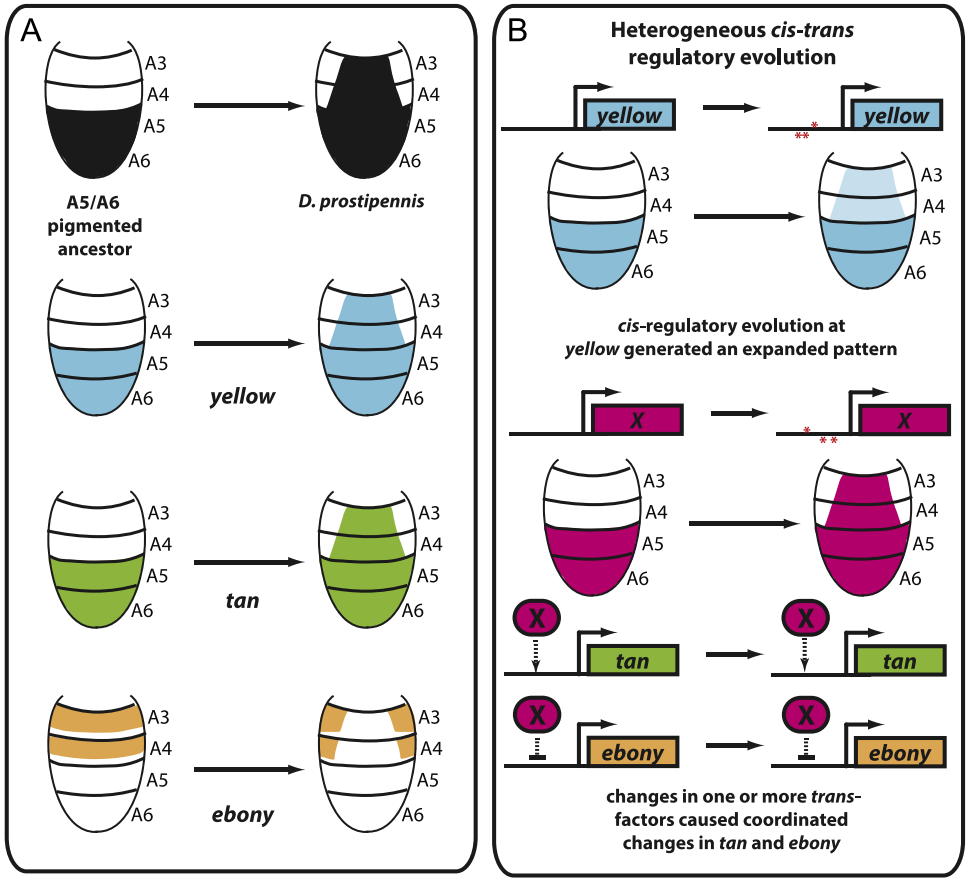

Fig. 6. Model for the coordinated evolution of a gene expression program. (A) Summary of our *in situ* hybridization results. Accompanying the expansion of black melanic pigment in the adult abdomen of *D. prostipennis*, we observed expanded expression pattern of two genes responsible for patterning dark pigment (*yellow* and *tan*) and the contraction of the yellow pigment promoting gene (*ebony*). (B) Our model for the coordinated evolution of the *D. prostipennis* phenotype depicts changes to the *cis*-regulatory region of *yellow* (denoted by red asterisks) that cause expanded expression. In parallel, a mutation in one or more *trans*-factor(s) (symbolized here as gene "*X*") drives the coordinated changes in *tan* and *ebony*. In the simplest form of the model, the expansion of gene *X* directly or indirectly activates *tan* in this new expanded region while simultaneously repressing *ebony*.

How many genes does it take to evolve reciprocal and correlated expression patterns?

Our finding that at a minimum, three enzymes of the pigment synthesis pathway have been modified in their expression profile to generate the *D. prostipennis* phenotype raises the question of how many genes were directly altered. An elaborate solution would necessitate *cis*-regulatory changes at all three genes to generate mutually exclusive and co-expressed patterns of pigmentation. However, we found that the activity of the *D. prostipennis ebony* and *tan cis*-regulatory sequences was nearly indistinguishable from the *D. takahashii* and *D. melanogaster* versions when placed in the *trans*-regulatory environment of *D. melanogaster* (Figs. 3 and 4). As a general rule, the pigmentation enzymes cannot cross-regulate each other – their expression patterns are unaltered in backgrounds in which the expression of one enzyme is up- or down-regulated. Thus, we must conclude that one or more changes in the transcriptional regulatory landscape of *D. prostipennis* must have occurred. Perhaps the simplest way for this to proceed is through a factor that is capable of directly or indirectly activating *tan*, while simultaneously down-regulating *ebony* (Fig. 6B, bottom). Alternatively, a more complex scenario would dictate changes to two or more *trans*-factors that expand *tan* expression, while increasing the extent of *ebony* repression in an independent manner.

Perhaps our most surprising finding was the drastic alterations to the *yellow* regulatory region of *D. prostipennis* (Fig. 5D), which mirrored the expanded expression of the gene (Fig. 2D′) – while *tan* and *ebony* genes appear to remain unaltered, *cis*-regulatory mutations accumulated in the *D. prostipennis* wb_element that precisely recapitulated its dorsally expanded expression in the A4 body segment (Fig. 5D). This result strongly implies that there are several ways to convergently evolve similar spatially restricted expression patterns in the *Drosophila* abdomen. As *yellow* evolved binding sites for factors that upregulated its dorsal expression and/or decreased its lateral expression in the A4 body segment, the *trans* factor (or factors) that evolved to alter *tan* and *ebony* expression likely derived altered patterns of expression similar or opposite to *yellow*. It is quite possible that this *trans* factor evolved an identical or similar set of inputs to those that arose at *yellow*.

However, our data do not exclude the possibility that additional factors upstream of *yellow* were also altered. In particular, the patterns driven by the *D. prostipennis yellow* reporter seem to be ectopic in the A2 and A3 segments as well as in females (S3), suggesting the existence of a repressing factor in *D. prostipennis* that is not present in *D. melanogaster*. It is a distinct possibility that the altered *trans* factor or factors upstream of *tan* and *ebony* also had a role in limiting the degree of ectopic expression of *yellow*. Future studies exploring the *trans*-regulatory landscape of patterning transcription factors and signaling pathways of *D. prostipennis* and *D. takahashii* could resolve this question.

The growing abdominal trans-regulatory landscape

Much of the observed diversity in *Drosophila* abdominal pigmentation has been correlated with the *bric-a-brac* (*bab*) transcriptional factors, which suppress dark coloration in the abdomen (Gompel and Carroll, 2003; Kopp et al., 2003, 2000; Rogers et al., 2013; Williams et al., 2008). Indeed, the absence of *bab* expression in the male posterior is correlated with the occurrence of sexually dimorphic pigmentation, as ancestrally monomorphic pigmented species express Bab monomorphically. Although Bab is a known repressor of *yellow* gene expression (Jeong et al., 2006), several lines of evidence suggest that it may not be a master-regulator of the reciprocal patterns of pigmentation gene expression present in the abdomen. First, the ectopic expression of *bab* in the male posterior is insufficient induce ebony expression (Rebeiz and Williams, unpublished observations). Second, *bab* expression does not always correlate with expansions and contractions of pigment patterns. Although *D. santomea* has lost abdominal pigmentation, Bab has maintained its sexually dimorphic profile of expression (Gompel and Carroll, 2003). In the *montium* lineage, *bab* remains restricted from the male A5 and A6 segments, although pigmentation is limited to the male A6 body segment in this group (Salomone et al., 2013). Finally, the male *D. prostipennis* Bab pattern does not correlate with its pigmentation phenotype and is nearly indistinguishable from that of *D. takahashii* (Williams and Salomone, unpublished observations). Therefore, we suggest that other factors must exist in the abdominal *trans*-landscape that regulate and pattern these three pigmentation genes. In a screen of three quarters of the transcription factors in the *Drosophila* genome for RNAi phenotypes, several factors were identified that caused pigmentation defects by altering both *tan* and *ebony* expression in reciprocal manners (Rogers et al., 2014). Thus, there exist several candidates that could foster coordinated evolution of pigment enzyme patterns while preserving mutual exclusivity.

Factors that may dictate the evolution of coordinated expression programs

Investigations into pigment pattern evolution across insect phyla indicate that a wide variety of paths exists for the coordinated evolution of gene expression programs. It is worth considering some examples of this phenomenon, and what properties may have favored one solution over another. In the highly varied coloration patterns of *Heliconius* butterfly wings, two major loci have been shown to underlie both the patterning and genetic diversification of pigment phenotypes. Alteration of red pigment pattern elements is caused by changes to the *optix* transcription factor, resulting in expression changes that perfectly pre-pattern red pigment deposition (Reed et al., 2011). As the varied expression of *optix* is completely due to changes at the *optix* locus, one can infer that the *trans*-regulatory landscape of the *Heliconius* wing is replete with patterning elements that could activate and sculpt a wide variety of *optix* expression patterns. Dark pigmentation in *Heliconius* wings shows reciprocal patterns of *ebony* and *tan* expression that correlate well with the spatial distribution of black wing patches (Ferguson et al., 2011). Changes in the patterning of melanic patches have been mapped to the *WntA* locus, which exhibits pigment correlated expression differences among morphs (Martin et al., 2012). In a functional genomic survey of gene expression in dissected *Heliconius* wings, dozens of genes were found to have pigment-associated gene expression (Hines et al., 2012), suggesting that many genes may participate in generating these characters. Hence, for both red ommochrome based pigments, and dark, melanin based pigments in butterflies, coordinated changes in expression programs appear to evolve through the alteration of “master regulator” genes that can direct the expression of multiple structural genes.

However, pigmentation traits in *Drosophila* have tended to be caused by differences in *cis*-regulatory elements of the pigmentation enzymes themselves (Gompel et al., 2005; Jeong et al., 2006; Prud'homme et al., 2006; Rebeiz et al., 2009a). This is not universal, however, (Kopp et al., 2000; Rogers et al., 2013; Werner et al., 2010; Williams et al., 2008), and may be strongly linked to the study of character loss in the aforementioned examples. The origination of a complex morphological trait, such as a pigmentation pattern may require several genes to fall into place, while the loss of the character may proceed through a small number of events that inactivate a gene or its regulatory elements. A second possible parameter that may channel how coordinated expression programs evolve is whether a master-regulator has

been established in the system. To date, the only known direct regulator of the pigmentation genes is the Hox factor Abd-B (Jeong et al., 2006), which seems unlikely to underlie the diverse male-specific expression patterns we observed (Figs. 1 and S1).

A third possibility is that pigmentation characters in *Drosophila* are fairly easy to manipulate, perhaps due to the relatedness of the pigments occurring across the body of the fly. The multiple shades of black, brown and yellow that adorn the *Drosophila* cuticle are all Tyrosine derivatives produced by the catecholamine synthesis pathway (Wright, 1993). As such, the simple mis-expression of *tan* and *yellow* is sufficient in the abdomen to convert its yellow colored cuticle to a black color (Jeong et al., 2008). In other systems, such as the butterfly wing, switching from one color type to another may require changes in many more genes since these pigments are derived from very different types of compounds (as well as structural colors). Hence, our results may reflect the relative ease of diverting pigment intermediates down simple alternate routes. The study of additional examples of coordinated evolution will be required to understand the forces shaping how complex developmental genetic programs tend to evolve.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.05.023.

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