Augmentation of a wound response element accompanies the origin of a Hox-regulated *Drosophila* abdominal pigmentation trait

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Abstract

A challenge for evolutionary research is to uncover how new morphological traits evolve the coordinated spatial and temporal expression patterns of genes that govern their formation during development. Detailed studies are often limited to characterizing how one or a few genes contributed to a trait's emergence, and thus our knowledge of how entire GRNs evolve their coordinated expression of each gene remains unresolved. The melanic color patterns decorating the male abdominal tergites of *Drosophila* (*D.*) *melanogaster* evolved in part by novel expression patterns for genes acting at the terminus of a pigment metabolic pathway, driven by *cis*-regulatory elements (CREs) with distinct mechanisms of Hox regulation. Here, we examined the expression and evolutionary histories of two important enzymes in this pathway, encoded by the *pale* and *Ddc* genes. We found that while both genes exhibit dynamic patterns of expression, a robust pattern of *Ddc* expression specifically evolved in the lineage of fruit flies with pronounced melanic abdomens. Derived *Ddc* expression requires the activity of a CRE previously shown to activate expression in response to epidermal wounding. We show that a binding site for the Grainy head transcription factor that promotes the ancestral wound healing function of this CRE is also required for abdominal activity. Together with previous findings in this system, our work shows how the GRN for a novel trait emerged by assembling unique yet similarly functioning CREs from heterogeneous starting points.

Keywords

cis-regulatory element; transcription factor; gene regulatory network; morphological evolution; Hox; Drosophila

Introduction

A major challenge for evolutionary developmental biology research is to explain how novel morphological traits come into existence through genetic changes and their underlying molecular mechanisms. The reigning paradigm for trait development involves the action of a gene regulatory network (GRN) in which a hierarchy of regulatory genes, encoding signaling pathway and transcription factor proteins, drive the expression of a set of realizator genes whose encoded proteins drive the physical manifestation of the trait (Davidson, 2006; Levine and Davidson, 2005). The expression patterns of a GRN's genes are specified by the binding of transcription factors to *cis*-regulatory elements (CREs) that function as enhancers or silencers of transcription (Arnone and Davidson, 1997; Gray and Levine, 1996; Johnson et al., 2015; Stanojevic et al., 1991). As GRNs are generally complex in realizator and regulatory gene number (Bonn and Furlong, 2008a), the origin of a novel trait requires an explanation regarding how the full set of realizator genes arrived at their appropriate patterns of expression (García-Bellido, 1975).

A multitude of possible scenarios could explain the origination of complex GRNs, and these differ in terms of their number and distribution of necessary genetic modifications. For instance, if a top-level regulatory gene of an ancestral GRN evolves a novel domain of expression, possibly by changing a single CRE, this could co-opt an entire GRN through the pleiotropic actions of this single gene (Monteiro and Gupta, 2016; Rebeiz and Tsiantis, 2017). In contrast, changes may be distributed throughout a GRN, involving the gain, modification, or loss of dedicated CREs for each realizator gene in the network. In order to distinguish whether the origins of novelty tend to emerge through such short or long evolutionary paths, it is necessary to characterize how GRNs and their constituent CREs evolve for a myriad of novel morphological traits in diverse lineages.

The coloration patterns adorning the cuticle of insects present ideal traits for investigation as a great diversity phenotypes exist within a tractable phylogenetic framework in which ancestral and derived character states can be reasonably inferred (Jeong et al., 2006; Wittkopp et al., 2003). One experimentally tractable coloration trait is the male-specific melanism of the dorsal tergites that cover the 5th and 6th abdominal segments of *Drosophila* (*D.*) *melanogaster* (Rebeiz and Williams, 2017). This dimorphic trait is suspected to have evolved from a monomorphic ancestral state in the *Sophophora* subgenus (Fig. 1A) (Jeong et al., 2006). In *D. melanogaster*, cuticle pigmentation requires the activities of several realizator genes whose protein products function in a tyrosine metabolic pathway that can produce yellow sclerotin or a black melanic cuticle (Fig. 1B) (Wright, 1987). In males, the realizator genes *yellow* and *tan* are highly expressed in the A5 and A6 segment epidermis underlying the developing tergites, expression of which is necessary for the formation of the black tergite color (Camino et al., 2015). In females, the realizator gene *ebony* is highly

expressed in the A5 and A6 segment epidermis (Rebeiz et al., 2009) which leads to broadly yellow tergites. The CREs responsible for the sex- and segment-specific expression patterns of *yellow*, *tan*, and *ebony* have been identified (Jeong et al., 2008; Rebeiz et al., 2009; Wittkopp et al., 2002), as well as some of the direct interacting transcription factors (Camino et al., 2015; Jeong et al., 2006; Roeske et al., 2018). Notably, *yellow* expression is directly activated by the Hox factor Abd-B binding to sites within the body element CRE, whereas the limitation of *tan* expression to 5th and 6th segments involves the direct binding of Abd-A and Hth to sites within the t_MSE to facilitate repression in the more anterior abdomen segments. Although *tan*, *yellow*, and *ebony* play essential roles in making black and yellow pigments, the pigment metabolism pathway involves several other key genes whose expression and regulation need to be understood within the context of this trait's development and evolution.

The first steps to forming catecholamine pigments are the conversion of Tyrosine to Dopa and then to Dopamine through the activity of Tyrosine Hydroxylase (encoded by the gene *pale*) (Neckameyer and White, 1993), and Dopa Decarboxylase (encoded by *Ddc*), respectively (Fig. 1B) (Wright, 1987). *pale* and *Ddc* differ from *yellow*, *tan*, and *ebony* in that their activities are seemingly needed to make both black and yellow pigments (Fig. 1B). Both proteins have been shown to accumulate transcripts before eclosion, but appear to be under translational control that governs the timing of tanning (Davis et al., 2007). However, the spatial distributions of *pale* and *Ddc* expression remain largely uncharacterized during pupal and adult stages when tergite pigmentation is developing, and the evolutionary origin of their CREs remain unresolved.

Here, we show that both *pale* and *Ddc* exhibit spatial and temporal patterns of expression during pupal and adult life stages in *D. melanogaster*. While *pale* expression has been broadly conserved among *Sophophora* species exhibiting both derived and ancestral pigmentation patterns, we show that high levels of epidermal *Ddc* expression evolved in the lineage of species with male-limited patterns of melanic pigmentation. This gain of abdominal *Ddc* expression requires the activity of a pleiotropic CRE that additionally regulates *Ddc* expression in the larval epidermis and in response to epidermis wounding (Mace et al., 2005; Scholnick et al., 1993). We find that the abdominal function of this CRE requires an ancestral binding site for Grainy head (Grh) necessary for its wound-response function. However, robust pupal abdomen CRE activity required changes elsewhere to this CRE. Together with previous results, our findings illustrate how a novel trait evolved through a lengthy evolutionary path, which involved independent gains of new expression patterns for an entire pathway of realizator genes through varied mechanisms.

Materials and Methods

Fly Stocks and Genetic Crosses

Fly stocks used in this study were maintained at 22°C on a previously published sugar food medium (Salomone et al., 2013). Species stocks used in this study were *D. melanogaster* (14021-0231.04), and *D. willistoni* (14030-0811.24) from the National Drosophila Species Stock Center, and *D. melanogaster yellow* and *white* (*yw*) gene mutant, *D. biarmipes*, and *D. pseudoobscura* stocks that were obtained from Dr. Sean B. Carroll. Mutant allele stocks used

to visualize abdomen pigmentation phenotypes were the null mutant y^1 (Bloomington Drosophila Stock Center or BDSC #169) allele for *yellow*, the hypomorphic t^5 (BDSC #133) allele for *tan*, and the null e^1 (BDSC #1658) allele for *ebony*. UAS-regulated RNA-interference (RNAi) stocks were generated by the Transgenic RNAi Project at Harvard Medical School, and acquired from the BDSC. These stocks express double stranded RNAs that specifically target *mCherry* (BDSC #35785), *grh* (BDSC #28820), *abd-A* (BDSC #28739), *hth* (BDSC #27655), *Ddc* (BDSC #51462), and *pale* (BDSC #65875). Experiments requiring ectopic expression of *Abd-B* in the A4 abdominal segment were achieved through crosses with a stock possessing the *Abd-B^{Mcp}* mutant allele (BDSC #24618). In order to use the GAL4/UAS system (Brand and Perrimon, 1993) in the dorsal abdomen midline, a stock with the genotype *pnr-GAL4/TM3, Ser^1* (BDSC #3039) was used that possesses a 3rd chromosome from which GAL4 is expressed in the dorsal midline pattern of the *pannier* gene (Calleja et al., 2000). In order to make qualitative, and in some cases quantitative, comparisons of reporter transgene activities, transgenes were integrated into the attP2 and/or 51D attP sites via phiC31 integrase-mediated transgenesis (Best Gene Inc.) (Bischof et al., 2007; Groth and Calos, 2004).

Gain- and loss-of-function experiments

To assess adult pigmentation phenotypes following reduction in either *pale*, *Ddc*, and *grh* expression, males from the respective *UAS-RNAi* stocks were crossed to *pnr-GAL4/TM3, Ser^1* virgin female flies. Adult progeny inheriting the *pnr-GAL4* chromosome and the *UAS-RNAi* transgene bearing chromosome express the double stranded (ds)RNA hairpin in the *pnr* gene's dorsal-medial pattern, including the pupal dorsal abdominal epidermis. The following crosses were made in order to investigate the effects of reduced transcription factor gene expression on the ability of the t_MSE and Ddc-MEE1 CREs to drive the expression of the *Enhanced Green Fluorescent Protein* (*EGFP*) reporter transgene in P14-P15(i) stage pupae. Virgin females from the RNAi stocks for *UAS-mCherry*, *UAS-abd-A*, *UAS-hth*, and *UAS-grh* were collected and separately crossed to males bearing a CRE reporter transgene in the 51D attP site of the 2nd chromosome (Bischof et al., 2007) and a *pnr-GAL4* bearing 3rd chromosome. As the *mCherry* gene does not exist in the *D. melanogaster* genome, expression of a dsRNA targeting this gene served as a negative control.

In order to compare the responsiveness of the Ddc-MEE1 and t_MSE CREs to *Abd-B* in pupae, adult males possessing the *Abd-B^{Mcp}* allele were crossed to virgin females possessing an *EGFP* reporter transgene under the regulatory control of either the t_MSE or the Ddc-MEE1 integrated into the 51D attP site (Bischof et al., 2007). As a negative control, similar crosses were made in which males from a *yw* stock that exhibit wild type expression of *Abd-B* (+) were utilized in place of the *Abd-B^{Mcp}* allele stock. EGFP reporter expression was assessed in P14-P15(i) stage pupae that inherited the reporter transgene and either the wild type or *Abd-B* mutant genotype.

in situ hybridization and immunohistochemistry

A previously describe protocol was used in the *in situ* hybridization studies (Jeong et al., 2008). In brief, digoxigenin labeled riboprobes for *Ddc* and *pale* were prepared through *in*

vitro transcription of PCR templates amplified from each species' genomic DNA (Table S1 for antisense probe primers and Table S2 for sense probe primers). Abdomens were dissected at the pupal developmental stages P10, P11, P12, P13, P14-15(i) and the newly eclosed adult fly stage P15(i). These different stages were identified by inspection for the presence and absence of various morphological markers (Ashburner et al., 2005) (Fig. S1). Probe hybridizations were made visible through the use of an anti-digoxigenin antibody (Roche Diagnostics), whose presence was detected by an alkaline phosphatase reaction using BCIP/NBT (Promega).

Dorsal abdomens were dissected for immunohistochemistry at the P12 and P14-P15(i) stages for *D. melanogaster* and *D. willistoni*. These stages are when we found *Ddc* transcription to be robust (P12) and declining P14-P15(i) in the *D. melanogaster* abdominal epidermis (Fig. 2). Qualitative comparisons of Grh expression patterns were made through immunohistochemical analysis using an antibody specific for Grh (Kim and Mcginnis, 2010). Abdomens were dissected to isolate the dorsal epidermis. Samples were fixed for 35 min in PBST (phosphate-buffered saline with 0.3% Triton X-100) with 4% paraformaldehyde, and then blocked in blocking buffer (PBST with 1% bovine serum albumin) for 1 hour at room temperature. The abdomens were then incubated overnight with guinea pig anti-Grh primary antibody at a dilution of 1:200 in PBST. After four washes in PBST and then an hour incubation in blocking buffer, specimens were incubated with goat anti-guinea pig Alexa Fluor 647 (Invitrogen) secondary antibody at a dilution of 1:500. After four washes in PBST, samples were incubated for 10 minutes in a 1:1 solution of glycerol mount (80% glycerol, 0.1M Tris pH 8.0) and PBST. Samples were transferred to glycerol mount, and finally situated between a cover slip and slide for imaging with a confocal microscope.

EGFP reporter transgenes and their quantitation

EGFP reporter transgenes were used to study the gene regulatory activity (Rebeiz and Williams, 2011) of sequences controlling *Ddc* expression and to make comparisons with the t_MSE that controls male-specific *tan* expression (Camino et al., 2015). All reporter transgene were made by cloning CREs into the *AscI* and *SbfI* restriction enzyme sites for the S3aG vector (Rogers and Williams, 2011). In this position each CRE is placed 5' of a minimal *hsp70* promoter and the coding sequence for the EGFP-NLS reporter protein (Barolo et al., 2004). The primer pairs used to amplify *D. melanogaster Ddc* sequences are presented in Table S3. The primer pairs used to amplify sequences orthologous to the *D. melanogaster* Ddc-MEE1 CRE are presented in Table S4. The details on the construction of the t_MSE EGFP reporter transgenes were discussed previously (Camino et al., 2015). Ddc-MEE1 transcription factor binding site mutant sequences (Fig. S2), and scanning mutant sequences (Fig. S3) were synthesized by GenScript USA Inc. Each synthesized sequence was flanked by an *AscI* and *SbfI* restriction enzyme site for subsequent cloning of the mutant CREs into the S3aG vector. Reporter transgenes containing truncated version of the *Ddc* upstream region, and those possessing Ddc-MEE1 scanning or binding site mutant sequences were each integrated in the attP2 site on the 3rd chromosome (Groth et al., 2000). The t_MSE, Ddc-MEE1, Ddc-MEE364, Ddc-MEE130, and orthologous Ddc-MEE1 CREs were integrated into the 51D attP site on the 2nd chromosome (Bischof et al., 2007).

Quantitative comparisons of the levels of EGFP reporter gene expression driven by sequences orthologous to the *D. melanogaster* Ddc-MEE1 were achieved following a previously described approach (Camino et al., 2015; Rebeiz and Williams, 2011; Roeske et al., 2018; Rogers et al., 2013; Rogers and Williams, 2011). In brief, for each transgene EGFP expression was imaged from four or five biological replicate specimens using a confocal microscope with settings for which few pixels were saturated when EGFP expression was driven by the *D. melanogaster* Ddc-MEE1. For each confocal image, a separate pixel value statistic was measured for the dorsal epidermis of either the A5 or A4 abdominal segment using the Image J program (Abràmoff et al., 2004). For each reporter transgene, the regulatory activity was calculated as the mean pixel value and standard error of the mean (SEM). CRE activities in the A5 abdominal segment for binding site mutant, scanning mutant, and orthologous Ddc-MEE1 sequences were normalized to the activity for the wild type Ddc-MEE1 in the same attP2 site, which was considered 100%. CRE activities in the A5 abdomen segment for the Ddc-MEE364 and Ddc-MEE130 were normalized to the activity for the wild type Ddc-MEE1 in the same 51D site, which was considered 100%. CRE activities in the A4 abdomen segment reported in the Abd-B^{Mcp} mutant background were normalized to the A4 activity of the species' CRE in an Abd-B wild type background, in which CRE activity was considered 100%.

Imaging of fly abdomens and experimental replication

Images of fruit fly abdomen pigmentation patterns were taken using an Olympus SZX16 Zoom Stereoscope and Olympus DP72 digital camera. Specimens were prepared from four day old flies. Projection images for EGFP-NLS reporter transgene expression were generated with an Olympus Fluoview FV 1000 confocal microscope and software. In each figure panel reporting a pigmentation pattern, gene expression pattern, or CRE activity, a representative image was selected from biological replicate specimens (n 4). In all figures where comparisons were made between images, each image was processed through the same sequence of modifications using Photoshop CS3 (Adobe).

DNA sequence representations and alignments

Sequence visualizations for the *Ddc* loci of *D. melanogaster* was made using the GenePalette tool (Rebeiz and Posakony, 2004; Smith et al., 2017). DNA sequence alignments of wild type, mutant, and orthologous Ddc-MEE1 sequences were made using the Chaos and Dialign software (Brudno et al., 2004).

Results

Melanic tergite pigmentation depends on *pale* and *Ddc* activity

The genetic and enzymatic pathway for the synthesis of the *D. melanogaster* cuticle pigments has been studied for several decades (Rebeiz and Williams, 2017; Wittkopp et al., 2003; Wright, 1987), though in recent years, much focus has been devoted to the expression and regulatory mechanisms of *tan*, *ebony*, and *yellow* genes which act at later steps in the pathway (Fig. 1B). Though the precise molecular mechanism of Yellow protein function remains unknown (Ferguson et al., 2011; Hinaux et al., 2018), the loss of *yellow* gene function results in a loss of black melanin pigments (Fig. 1F and F'). *ebony* encodes a

protein with NβAD synthetase activity which shunts Dopamine to form a yellow colored NβAD sclerotin. The loss of *ebony* function results in a broadly melanic phenotype (Fig. 1H and H'). *tan* encodes an enzyme with NβAD hydrolase activity, which converts NβAD back to Dopamine (True et al., 2005). Reduced *tan* activity results in a broad loss of melanin pigments (Fig. 1G and G'). *pale* encodes an enzyme with Tyrosine hydroxylase activity, which is the initial step in the pigmentation pathway converting Tyrosine to Dopa. RNA-interference (RNAi) of *pale* expression in the dorsal midline of *D. melanogaster* results in a dramatic loss of melanic pigments and to a lesser extent yellow cuticle color (Fig. 1D and D'). *Ddc* encodes an enzyme with Dopa decarboxylase activity and is responsible for converting Dopa to Dopamine. RNAi for *Ddc* in the dorsal midline results in a stark reduction in melanic pigments in the abdominal tergites (Fig. 1E and E').

Collectively, these results show that *pale* and *Ddc* are essential for the formation of the sexually dimorphic pattern of *D. melanogaster* abdomen pigmentation. The dramatic loss of black colored cuticle following *Ddc* RNAi indicates that most black color is that of Dopamine melanin rather than Dopa melanin (Fig. 1B). *ebony*, *tan*, and *yellow* have been established to be expressed in complex spatial, temporal, and sex-specific manners. Thus, we sought to reveal whether the expression patterns for *pale* and *Ddc* are similarly complex.

The spatial and temporal pattern of abdominal *pale* expression

During the approximately 100 hour time course of *D. melanogaster* pupal development at 25°C, pupae transition through roughly 15 distinct stages that can be identified by morphological markers (Fig. S1) (Ashburner et al., 2005). By the P10 stage, *pale* is expressed robustly in the abdomen mechanosensory bristle cells, whereas little to no expression is seen in the surrounding epidermis (Fig. 2A and A'). This pattern of expression is maintained through the P12 stage when some bristles have developed clear projections (Fig. 2B and B'). By the P14-P15(i) stage, *pale* expression has dramatically increased in the abdominal epidermis region underlying where tergites will form, and is especially elevated in the A5 and A6 segments. Expression of *pale* is comparatively absent from the epidermal cells that will be covered by flexible un-pigmented inter-segmental membrane that will ultimately fold underneath each tergite (Fig. 2C and C'). This epidermal pattern of expression is sustained in newly eclosed, P15(ii) stage, flies (Fig. 2D and D'). Collectively, these results show that pale undergoes a complex temporal and spatial pattern of expression like *yellow*, *tan*, and *ebony*. However, *pale* differs from the other pigmentation genes in that its expression is not conspicuously dimorphic.

The spatial and temporal pattern of abdominal *Ddc* expression

By the P10 stage, *D. melanogaster* *Ddc* is expressed robustly in the abdomen mechanosensory bristle cells, whereas the surrounding epidermis lacks detectible expression (Fig. 2F and F'). By the P12 stage, *Ddc* expression has dramatically increased in the abdominal epidermis region underlying where tergites will form, notably in the A3–A6 segments, while expression is comparatively absent from the epidermal cells that will be covered by flexible un-pigmented inter-segmental membrane (Fig. 2G and G'). By the P14-P15(i) stage, *Ddc* expression remains in the abdominal epidermis though the level of expression has declined (Fig. 2H and H'). In newly eclosed flies, *Ddc* expression has

become difficult to detect (Fig. 2I and I'). Collectively, these results show that *Ddc* undergoes a complex temporal and spatial pattern of expression like *yellow*, *tan*, and *ebony*. However, like *pale*, *Ddc* expression is not conspicuously dimorphic. As the pattern of *D. melanogaster* tergite pigmentation represents a novel trait that evolved from a monomorphic ancestral state, we were curious whether the *Ddc* and *pale* expression patterns were modified during this trait's origins.

Conserved *pale* expression is a fixture of evolved pigmentation traits and GRNs

The working model for pigmentation evolution in the *Sophophora* subgenus places a monomorphic pattern of tergite pigmentation as ancestral, and dimorphic pigmentation being a derived state (Jeong et al., 2006; Rebeiz and Williams, 2017). Moreover, many species within the *obscura* species group, including *D. pseudoobscura* (Fig. 1A), are darkly pigmented across the entire abdomen. It has been shown that *yellow* and *tan* expression correlate with these patterns of pigmentation, and *ebony* expression has an inverse correlation (Camino et al., 2015; Jeong et al., 2008; Johnson et al., 2015; Ordway et al., 2014; Rebeiz et al., 2009). We sought to reveal whether the earliest acting gene in the *Drosophila* pigmentation pathway, *pale*, has similarly evolved diverse patterns of expression to shape the visible pigmentation patterns seen among *Sophophora* (Fig. 1A).

We focused our attention on the P10, and P15(ii) developmental stages for which we found *D. melanogasterpale* expression to transition from a mechanosensory-specific expression pattern to a pattern including the abdominal epidermis. We observed that expression at the P10 and P15(ii) stages were conserved between species with dimorphic and monomorphic patterns of pigmentation (Fig. 3). This outcome suggests that the expression pattern for *pale* has been a conserved feature of the evolving pigmentation GRNs for abdominal tergite pigmentation.

Ddc is broadly expressed in the abdomen epidermis of highly pigmented species

The conserved abdominal epidermis pattern of *pale* expression represents a departure from the evolved patterns observed with *yellow*, *tan*, and *ebony*. We sought to determine whether the second gene in the pigmentation pathway, *Ddc*, is characterized by a conserved pattern of abdominal epidermis expression. We focused our attention on the P10 and P12 stages of pupal development where we respectively observed a conspicuous pattern of expression in the mechanosensory bristle cells and abdominal epidermis for *D. melanogaster* (Fig. 2).

At the P10 stage, robust mechanosensory expression was found in all species evaluated (Fig. 4A–D and A'–D'), consistent with their melanic appearance. At the P12 stage, *Ddc* expression occurs in a monomorphic pan-abdomen epidermal pattern for *D. biarmipes* and *D. pseudoobscura* (Fig. 4E–4G and E'–G'). *D. biarmipes* has a male-specific pattern of tergite pigmentation like *D. melanogaster*, and *D. pseudoobscura* has a monomorphic melanic pattern that spans the tergites of the A1–A6 segments (Fig. 1A). Interestingly though, at the P12 stage we detected little to no *Ddc* expression in the abdominal epidermis of *D. willistoni* (Fig. 4H and H'). Expanding our search to a range of developmental stages, we failed to detect strong epidermal expression in *D. willistoni*, despite the clear expression detected in bristle cells (Fig. 5). Unlike the other species evaluated, *D. willistoni* lacks

notable pigmentation on its abdominal tergites (Fig. 1A), and this pigmentation pattern is considered to descend from a lineage bearing the ancestral pattern from which dimorphism evolved (Jeong et al., 2006). These results suggest that the origin of dimorphic pigmentation involved the gain of a robust pattern of *Ddc* expression in the abdominal epidermis. Thus, we next sought to understand how *Ddc* expression is regulated in the abdominal epidermis and elucidate how this expression pattern evolved.

A multifunctional CRE drives *Ddc* expression in the abdominal epidermis

The *Ddc* locus resides on *D. melanogaster* chromosome 2, between the genes *l(2)37Cc* and *CG10561* (Fig. 6A). *Ddc* has three transcribed exons, although a splice variant exists that includes an additional non-coding exon situated in the first intron. As a first pass at identifying CREs active in the abdomen during development and pigmentation pattering, we crossed virgin females carrying a UAS-GFP-nls transgene to males of the GMR61H03 and GMR601F07 GAL4 lines (Pfeiffer et al., 2008). These GAL4 lines respectively possess a 1,692 base pair (bp) piece that spans most of the sequence between the *Ddc* 1st exon and the last exon of the upstream *l(2)37Cc gene*, and a 1,115 bp sequence spanning the entire *Ddc* 2nd intron (Fig. 6A). In pupae inheriting the UAS-GFP-nls and GMR61H03-GAL4 transgenes, we observed GFP expression in the abdominal epidermis of the A1–A6 segments which was noticeably elevated in more posterior segments (Fig. 6B). On the other hand, GMR61F07-GAL4 drove GFP expression in the developing mechanosensory bristle cells (Fig. 6C). Collectively, these patterns of GFP expression recapitulated our observed patterns *Ddc* mRNA expression detected by *in situ* hybridization experiments (Fig. 2), and demonstrates that the two phases of pupal abdomen expression are under the control of multiple modular CREs.

To examine the molecular basis for the evolved patterns of *Ddc* expression in the abdominal epidermis, we focused on the GMR61H03 fragment. Generating direct fusions of sub-fragments to *Enhanced Green Fluorescent Protein* (*EGFP*) reporter gene, we found this *Ddc* sequence, named Ddc-MEE, to drive a similar abdominal epidermis pattern of reporter expression (Compare Fig. 6D to B). To better resolve which sequences within this Ddc-MEE region drives the pupal abdominal epidermis reporter expression, we created three reporter transgenes that subdivided this region (Fig. 6A).

We found that the reporter gene containing the promoter-proximal region (Ddc-MEE1) drives EGFP expression in a pattern indistinguishable from that of the Ddc-MEE (compare Fig. 6E to B and D). EGFP expression was more robust in the male abdomen than in the female abdomen when the Ddc-MEE1 reporter transgene resides in the attP2 insertion site, though this disparity was less dramatic when the same reporter resides in the 51D attP site (Fig. S4). This modest dimorphism may have at least two explanations. The first is that the increased activity is due to the reporter transgene vector exhibiting a dosage-compensation boost in males due to the vectors inclusion of the *mini-white* version of the X-chromosome *white* gene (Ashburner et al., 2005; Qian and Pirrotta, 1995). The other possibility is that CRE reporter transgenes make expression differences more apparent than *in situ* hybridization. We found the central (Ddc-MEE3) region to lack any noteworthy pupal abdomen regulatory activity (Fig. 6G). In contrast, the distal (Ddc-MEE2) region drove

strong epidermis activity (Fig. 6F), which poorly matched expression of the larger fragment or endogenous expression. We interpret this fragment to either be a secondary CRE (Hong et al., 2008; Lagha et al., 2012), a CRE regulating another gene, or artefactual activity. Since the Ddc-MEE1 region generally drives a pattern of expression similar to the evolved pattern of *Ddc* expression (Fig. 4), we next sought to determine how this CRE is regulated and evolved.

The Ddc-MEE1 pigmentation patterning activity is regulated by Grainy head

The Ddc-MEE1 sequence 5' of the *Ddc* first exon possesses binding sites (Fig. 7A) that contribute to multiple previously described regulatory activities. Within this sequence, a single ecdysone response element (EcRE) is required for the spike in *Ddc* expression that occurs at puparation (Chen et al., 2002). For the wound response activity, binding sites for the AP-1, CREB-A, and Grh transcription factors were necessary (Mace et al., 2005). Hence, we referred to this sequence as the Ddc-Multifunctional Epidermis Element 1 or Ddc-MEE1. We sought to determine whether any of these binding sites were required for the tergite pigmentation patterning activity of *Ddc*, by introducing the previously characterized mutations that disrupt these sites into the Ddc-MEE1. We observed no noticeable alteration in Ddc-MEE1 activity when the EcRE site was mutated, nor when the two AP-1 and the CREB-A sites were mutated (Figs. S2 and S5). However, activity of the Ddc-MEE1 was reduced to 49±3% of the activity of the wild type element when the two Grh sites were mutated (compare Fig. 7C to B).

To further validate a role for *grh* in tergite pigmentation patterning, we used the GAL4/UAS system (Brand and Perrimon, 1993) to drive a dsRNA specific for *grh* in the dorsal midline region. Compared to the normal tergite pigmentation phenotype observed when RNAi targeted the negative control *mCherry* gene, pigmentation was greatly reduced upon *grh* knockdown (compare Fig. 8A and A'). Relatedly, when we tested the Ddc-MEE1 reporter under these conditions, its activity was dramatically reduced (compare Fig. 9J to 9I). Collectively, these data reveal that melanic tergite pigmentation is positively-regulated by Grh, a result that can be explained by the direct regulation of *Ddc* in the pupal abdomen through one or both of the pleiotropic binding sites in the Ddc-MEE1 CRE.

Previously, the *Ddc* wound response element was resolved down to the immediate 472 bp upstream of the gene's transcriptional start site (Mace et al., 2005). We wanted to determine whether the wound and tergite pigmentation patterning activities shared additional function-driving sequences, or whether sharing was limited to the Grh binding sites. We created a set of 14 scanning mutant Ddc-MEE1 sequences, in which a different block of ~100 bp was mutated at every other bp by a non-complementary transversion mutation (Figs. 7A, S3, and S6) (Camino et al., 2015; Rogers et al., 2013). For twelve of the fourteen mutant CREs, we saw no conspicuous alteration in the expression of the EGFP reporter gene (Fig. S6). However, reporter expression observed in the abdominal epidermis for the Ddc-MEE1 CREs with either the SM11 and SM12 mutations were reduced respectively to 43±5% and 49±7% of the activity measured for the wild type element (compare Fig. 7D and E to B). The sequences disrupted by SM11 and SM12 mutations include sequences necessary for the wound response activity, and the more evolutionarily conserved of the two Grh biding sites

(Mace et al., 2005) in the larger wound element CRE (Fig. S7). As the SM11 and SM12 sequences were necessary for the pupal abdomen epidermis regulatory activity, we tested whether these sequences were sufficient in the context of two truncated versions, named the Ddc-MEE364 and Ddc-MEE130 sequences (Figs. 6E, H and I, and S8). The Ddc-MEE364 contains the non-mutant sequence for the SM11 and SM12 regions. This truncated sequence retained the abdominal epidermis pattern of activity, albeit with modestly-reduced levels of EGFP reporter expression compared to the larger Ddc-MEE1 sequence (82±3%, Fig. S8). The Ddc-MEE130 sequence contains the non-mutant sequence for the SM11 region and the more conserved Grh binding site, but lacks the adjacent EcRE. Similar to the Ddc-MEE364, the Ddc-MEE130 also had a moderated activity compared to that of the Ddc-MEE1 sequence, though it's pattern resembled the Ddc-MEE1 and Ddc-MEE364 reporters (86±8%, Fig. S8). These experiments localize the bulk of the Ddc-MEE1's patterning and activity to the MEE130 region, though additional sequences elsewhere within the Ddc-MEE1 are required for full activity. We next sought to investigate which transcription factors in addition to Grh shape the pupal abdomen activity of the Ddc-MEE1 CRE.

The abdominal epidermis activity of the Ddc-MEE1 is Hox-regulated

Previous studies revealed that the *D. melanogaster* A5 and A6 tergite pigmentation and expression of *yellow* and *tan* genes are regulated by Hox transcription factors and their cofactors (Jeong et al., 2006; Rogers et al., 2014). We were curious whether Ddc-MEE1 regulatory activity is Hox-regulated as well. *Abd-B*, the *Hox* gene with the most posterior-limited expression, is present in the A5 segment and more posterior segments (Kopp and Duncan, 2002; Wang and Yoder, 2012). In the *Abd-B^{Mcp}* mutant background, Abd-B expression additionally includes the A4 segment. Like the t_MSE, which controls *tan* expression, ectopic A4 segment reporter expression occurs when the Ddc-MEE1 is assayed in the *Abd-B^{Mcp}* background, indicating that this element is downstream of *Abd-B* (Compare Fig. 9B to A for *tan*, and H to G for *Ddc*).

Ddc expression and Ddc-MEE1 activity extends anterior of the A5 segment, and thus other transcription factors must govern expression and CRE activity in these segments. Plausible candidates include the Hox protein Abd-A which is expressed up through the A2 segment (Kopp and Duncan, 2002; Rogers et al., 2014) and the Hox cofactor Hth which directly represses t_MSE activity in the anterior abdomen segments (Camino et al., 2015). Given the similarities of *Ddc* expression with *tan*, we compared the expression of the Ddc-MEE1 with that of the t_MSE under RNAi conditions for factors that disrupt t_MSE function. The t_MSE activity is reduced upon *abd-A* RNAi (Fig. 9E), and shows ectopic expression in the A3 and A4 segments when *hth* expression is antagonized by RNAi (Fig. 9F). In contrast, we observed only minor reductions in midline reporter expression of the Ddc-MEE1 when *abd-A* was reduced (Fig. 9K). Similarly, RNAi for *hth* did not increase the A3 and A4 segment reporter expression of the Ddc-MEE1 (Fig. 9L). These results suggest that the pupal abdominal epidermis regulatory activity of the *D. melanogaster* Ddc-MEE1 depends upon a unique set of factors, compared to the t_MSE. The discovery that *Ddc* expression is Hox-regulated inspired us to examine whether the gain in pupal abdomen *Ddc* expression for *D. melanogaster* and its melanic relatives (Fig. 4) involved *cis*-regulatory evolution at the Ddc-MEE1.

The activity of the Ddc-MEE1 was shaped by *cis*-evolution

To this point, our results suggested that the transition from an ancestral monomorphic pigmentation to derived melanic patterns involved the gain of a robust pattern of epidermal *Ddc* expression during the P12 developmental stage (Fig. 4). One possible explanation for the derived expression pattern is through the gain or modification of a CRE activity driving *Ddc* expression. To test this possibility, we investigated the regulatory activities of sequences orthologous to the *D. melanogaster* Ddc-MEE1 from species with dimorphic (*D. biarmipes*), monomorphic melanic (*D. pseudoobscura*), and monomorphic non-melanic (*D. willistoni*) patterns of tergite pigmentation (Fig. 10A–D). We saw that each of the orthologous sequences were capable of driving reporter expression in the abdominal epidermis of *D. melanogaster* (Fig. 10A'–D'), indicating that the origin of the P12 stage epidermal *Ddc* expression was not due to the evolution of an altogether new CRE activity. Importantly though, EGFP reporter expression driven by the *D. willistoni* sequence was consistently more modest in biological replicate specimens (Fig. 10D'). Quantification of EGFP intensity in the A5 segment revealed that the *D. willistoni* CRE drove 40±1% of the *D. melanogaster* Ddc-MEE1 activity (100±7%; compare Fig. 10D' to A'). In contrast, the *D. biarmipes* and *D. pseudoobscura* activities respectively were 111±3% and 114±2% of the *D. melanogaster* CRE's activity (Fig. 10B' and C'). In a genetic background with ectopic *Abd-B* expression in the A4 abdomen segment, the activity of the *D. melanogaster* Ddc-MEE1 increased to 180±14% of the activity in the A4 segment of a *Abd-B* wild type background (Fig. S9). In comparison, the activity of *D. willistoni* sequence orthologous to the *D. melanogaster* Ddc-MEE1 increased to 148±10% A4 segment activity of the *D. willistoni* CRE in a wild type background (Fig. S9). These results are consistent with a scenario in which the enhanced expression of *Ddc* in the pupal abdomen epidermis of *D. melanogaster* involved *cis*-evolution. This regulatory evolution seems to have included an increased responsiveness of the Ddc-MEE1 to Hox-regulation compared to the antecedent CRE.

While our results implicate a role for *cis*-evolution in shaping the activity of the Ddc-MEE1 CRE, it is also possible that changes to the expressions or activities of *trans*-regulators were additionally required. One plausible candidate would be spatially and/or temporally expanded expression of Grh, the direct regulator of the Ddc-MEE1. However, we observed a conserved pattern of pan-abdominal epidermis Grh expression in the abdomens of *D. melanogaster* and the monomorphic *D. willistoni* (Fig. 8). Thus, future studies are needed to resolve whether *trans*-evolution played a role in the derived expression pattern of *Ddc*.

Discussion

Our findings illustrate how an important realizator gene was recruited to a novel trait through the alteration of a presumably ancient CRE that functions in an entirely unrelated task. By characterizing the expression of two genes whose protein products catalyze early steps of melanin synthesis, we found that strong *pale* expression is conserved broadly across the *Sophophora* subgenus, while high levels of *Ddc* expression were only observed in species that had derived dark melanic pigments. We uncovered how abdominal *Ddc* expression in *D. melanogaster* is driven by a pleiotropic CRE that plays a presumably ancient role in activating *Ddc* expression following epidermal wounding (Fig. 11A).

Notably, a Grh binding site required for wound healing is also required for its abdominal function. Comparative analysis of this CRE revealed that its augmented pigment associated activity evolved more recently in the lineage of species with melanic abdomens. These results contribute to a body of literature showing that the origin of dimorphic pigmentation required *cis*-evolution at several realizator genes in the network (Figure 11B), providing an example in which an entire network was assembled through widespread *cis*-regulatory modifications.

The novel deployment of a realizator gene pathway by widespread CRE evolution

The development of most well-studied animal morphological traits relies upon dozens of regulatory genes that specify the spatial and temporal expression of pathways of realizator genes (Bonn and Furlong, 2008b; Levine and Davidson, 2005). One of the most difficult challenges in evolutionary biology is to develop molecular explanations of how such vast networks of regulators and realizators emerge to control novel traits. A critical feature that must be resolved is whether these novel traits are built up gradually through evolution at each realizator, or if networks evolve primarily through the co-option of large batteries of realizators from one developmental setting to another by expressing their upstream regulators in new locations (Monteiro and Gupta, 2016; Rebeiz et al., 2015). To address this distinction, the pigmentation of the *Drosophila* abdomen is one of the most extensively studied traits for which the evolutionary trajectories of key components of the network have been traced. This study expands a growing perspective of how its network was assembled.

The current favored model for pigment evolution in the *Sophophora* subgenus places monomorphic, non-melanic pigmentation as the ancestral character state, as exemplified by *D. willistoni*, and male-specific melanic pigmentation as the derived state, as seen for *D. melanogaster* and *D. biarmipes* (Fig. 1A) (Rebeiz and Williams, 2017). In the *obscura* species group, a broadly monomorphic melanic character state evolved. The expression of *yellow, tan*, and *ebony* evolved to fit these different pigmentation patterns. For *D. willistoni*, the dorsal abdominal epidermis experiences little to no *yellow* and *tan* expression, whereas expression occurs robustly throughout the *D. pseudoobscura* abdomen and in the male-specific pigmented regions of *D. melanogaster* (Camino et al., 2015). Thus, the origin of melanic phenotypes required gains in *yellow* and *tan* expression. In this study, we show that while *pale* expression throughout the abdominal epidermis has maintained high levels of ancestral expression, *Ddc* evolved a robust pattern of expression in the abdomen epidermis in the lineage of melanic species. Thus, a critical question is whether this network evolved its associated expression domains through co-option or independent evolutionary changes.

If the origin of male-specific tergite pigmentation in the *D. melanogaster* lineage originated via a co-option mechanism, we would anticipate two hallmark signs of this event. First, co-option would likely involve a master regulator whose activity is sufficient to deploy all of the underlying regulatory and realizator genes. Second, we would expect these CREs to all share an ancestral activity in a second tissue or developmental setting from which the abdomen melanization function was co-opted (Glassford et al., 2015). The case we presented here of the Ddc-MEE1 illustrates a clear ancestral function at Ddc that is unique among genes of the pigmentation pathway. To our knowledge, none of the other CREs in the pathway are

associated with a wound response function. Further, we have yet to identify a master regulator of the network that would make sense as a source of the co-option event. Of all the transcription factors thus far identified in the abdominal pigmentation network, the most likely master regulator is *Abd-B*. CREs of four realizator enzymes of the network respond to *Abd-B* in *trans* (Camino et al., 2015; Jeong et al., 2006), and in the case of *yellow*, Abd-B is a direct regulator (Jeong et al., 2006). However, the major problem with considering Abd-B as a master regulator that mediated the redeployment of this network, is that its pattern of expression is likely ancestral, and an Abd-B dependent ancestral network that drives all of these genes elsewhere in the posterior of the embryo, larvae, or pupae seems unlikely. Indeed, upon discovering the direct binding of the Bric-á-brac (Bab) transcription factors to the CRE controlling abdominal epidermis *yellow* expression, we found no evidence of co-option from an ancestral CRE function (Roeske et al., 2018). Thus, considering the lack of a shared ancestral network or master regulatory gene, we propose that the network of pigmentation realizator CREs was assembled in a piecemeal fashion. The path by which *Ddc* independently evolved its abdominal activity is a curious one.

Gene expression evolution through the augmentation of a pleiotropic CRE activity

To understand how GRNs of regulators and realizators emerged to generate novel traits, we must precisely examine how their CRE activities originated. Several mechanisms have been posited, including the gain of a CRE by transposition, the *de novo* evolution of a CRE activity by gains in transcription binding sites, and the co-option of an existing CRE with an ancestral function (Rebeiz et al., 2015). Problematically though, differences in traits between closely-related species often represent losses, and experimental models for trait gains often require comparisons between more distantly-related taxa whose *cis*-regulatory regions may be difficult to compare due to extensive sequence divergence.

The gain of a fruit fly male-specific wing pigment spot and abdomen tergite pigmentation have been two productive models for investigating the origins of CREs associated with novel traits. In *D. biarmipes*, a spot of pigmentation on the wing requires the expression of *yellow* that is driven by a CRE that possesses binding sites for the activator Dll and the repressor En (Arnoult et al., 2013; Gompel et al., 2005). The spot CRE resides adjacent to a wing element CRE that drives a low level of *yellow* expression throughout the developing wing, and comparative analyses of this region suggested that the spot CRE evolved from this weakly active element. Similarly, the origin of the *D. melanogaster* abdominal tergite pigmentation required gains of male-specific patterns of *yellow* and *tan* expression (Camino et al., 2015; Jeong et al., 2008, 2006). In *D. melanogaster*, *yellow* expression is driven by an Abd-B and Bab regulated CRE known as the body element (Jeong et al., 2006; Roeske et al., 2018), and *tan* is driven by a Hox and Hox cofactor regulated CRE known as the t_MSE (Camino et al., 2015). The origin of these CRE activities remain difficult to resolve, as the surrogate for the ancestral trait, *D. willistoni*, lacks these CRE activities and any sequence with noteworthy conservation. Thus, the genetic events and molecular details responsible for the origin of the *yellow* body element and the *tan* t_MSE remain nebulous.

In contrast, we found clear evidence for the evolutionary path taken by *Ddc*, as it gained a robust pattern of abdominal epidermis expression in melanic species. Unlike the CREs of

yellow and *tan*, the orthologous sequence of the Ddc-MEE1 is highly conserved (Fig. S7), and the ancestral function can be reasonably inferred as this region is known to drive *Ddc* expression following epidermal wounding between fruit fly species more distantly-related than those studied here (Mace et al., 2005). Moreover, some of the molecular details regarding the evolution of the Ddc-MEE1 can be inferred. Both the wound response (Mace et al., 2005) and abdominal epidermis activities require a conserved binding site for the Grh transcription factor (Fig. S7). This indicates that the ancestral wound response CRE was modified, resulting in the augmentation of its pleiotropic function in tergite pigmentation (Fig. 11A). While the precise details on the mechanism of augmentation remains to be elucidated, part of the explanation requires the gain of an increased responsiveness to the *Hox* gene, *Abd-B*. Importantly, our work provides an example whereby part of a trait's origin required the gain of a realizator gene's expression through the modification of an ancient CRE for an augmented pleiotropic use. Our ability to discern an evolutionary past to this element highlights how different CREs, even in the same network, will contrast greatly in their rates of divergence. Finally, our findings reinforce how CRE activities can emerge from unexpected developmental contexts that provide a rich molecular explanation for the origin of traits and the CREs that enable them.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *Drosophila melanogaster pale* and *Ddc* expression are temporally and spatially dynamic

- *Ddc* expression is augmented in species with derived melanic pigmentation patterns

- *Ddc* expression is driven by an enhancer with an ancestral wound response activity

- Augmented *Ddc* expression evolved through modifications in the wound response enhancer

- A novel pigmentation trait required widespread enhancer evolution in realizator genes
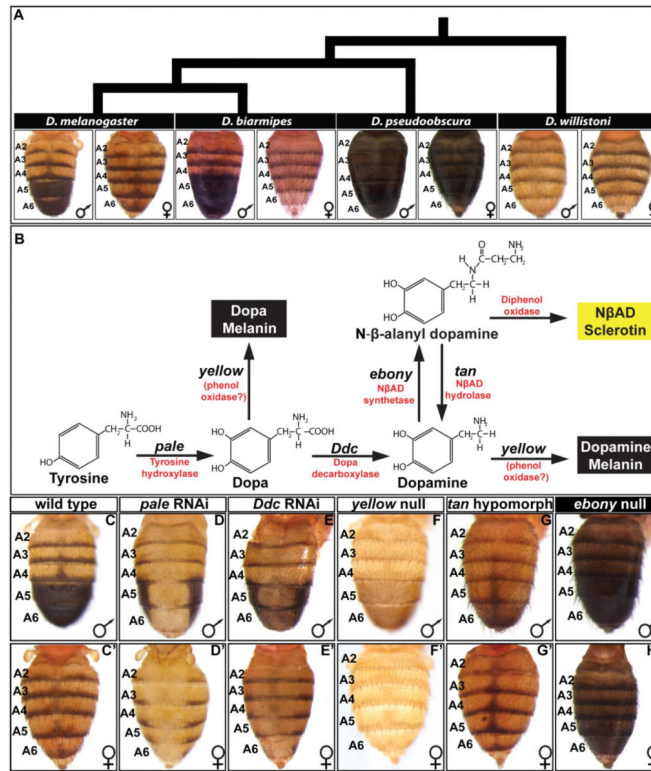
Fig. 1. Pigmentation phenotypes and enzymatic pathway in the *Sophophora* subgenus.
(A) Extant species that represent the inferred diversification of abdominal pigmentation from an ancestral monomorphic state to a derived dimorphic state in the *Sophophora* subgenus. The tergites covering the A5 and A6 abdominal segments of *D. melanogaster* and *D. biarmipes* are sexually dimorphic for a melanic pigment color, with the male tergites being fully pigmented and pigmentation limited to posterior stripes in females. The more distantly related species such as *D. pseudoobscura* and *D. willistoni* are monomorphic, though differ greatly in the extent of their melanization. (B) The production of yellow and black cuticle depends on the activity of a metabolic pathway converting Tyrosine into NβAD sclerotin or Dopa/Dopamine melanin. (C and C') The wild type abdomen pigmentation pathway includes a male-specific pattern of fully melanic A5 and A6 segment tergites. (D and D') RNA-interference for *pale* results in a near complete absence of black cuticle and a reduction in yellow cuticle color. (E and E') RNA-interference for *Ddc* results in a stark reduction in black cuticle color and a reduction in yellow color. (F and F') The null allele phenotype for *yellow* is a loss of black cuticle color. (G and G') The hypomorphic allele phenotype for *tan* is a reduction in black cuticle color though the stripe and midline spot region pigmentation remains. (H and H') The null allele phenotype for *ebony* is a broadening of black cuticle to all abdomen segments. RNA-interference was achieved by driving a UAS-regulated dsRNA transgene for *pale* and *Ddc* by the GAL4 transcription factor that was expressed in the dorsal-medial pattern of the *pnr* gene.
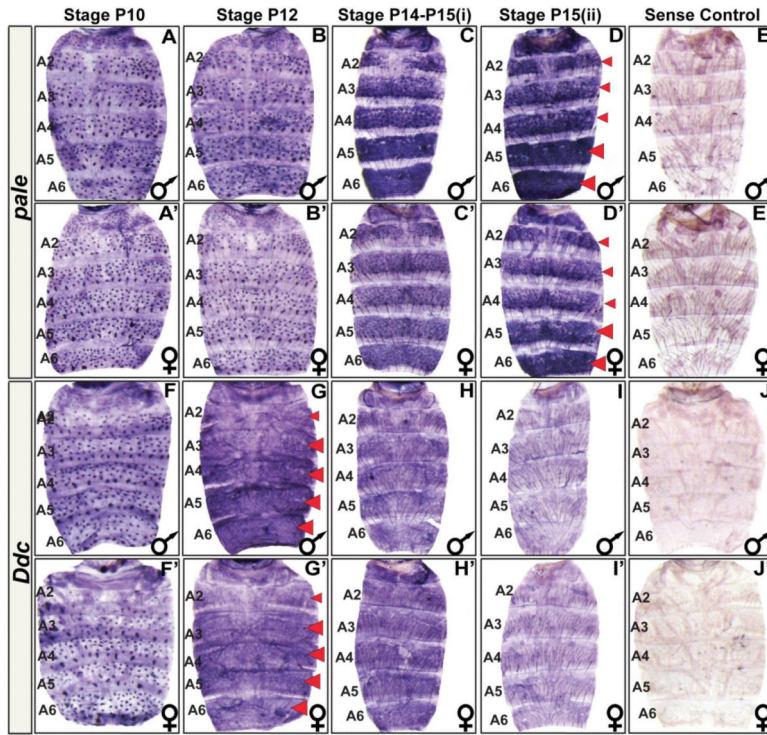
Fig. 2. The spatial and temporal expression pattern of *D. melanogaster pale* and *Ddc*.
(A–J) Samples for *D. melanogaster* males and (A'–J') females. (A and A') *pale* is expressed in the mechansosensory bristle cells as early as the P10 stage of pupal development. (B and B') Mechanosensory cell expression continues through the P12 stage. (C and C') By the P14-P15(i) stage, *pale* expression has dramatically increased in the abdominal epidermis. Most notably in the A5 and A6 segments and in the epidermal cells underlying where tergites will form. (D and D') The epidermis pattern of *pale* expression can still be detected in newly eclosed flies. (E and E') A sense probe control shows the background pattern of signal observed in an eclosed fly. (F and F') *Ddc* is expressed in the mechansosensory bristle cells as early as the P10 stage of pupal development. (G and G') By the P12 stage, *Ddc* expression has dramatically increased in the abdominal epidermis, most notably cells in the A3–A6 segment regions underlying where tergites will form. (H and H') By the P14-P15(i) stage, *Ddc* expression persists but at an apparently reduced level. (I and I') Epidermis expression has become difficult to detect in newly eclosed flies. (J and J') A *Ddc* sense probe control shows background signal observed in eclosed fly. Red arrowheads indicate stages and segments with conspicuous expression detected in the epidermis. These stages were prioritized for investigations of expression in related fruit fly species.
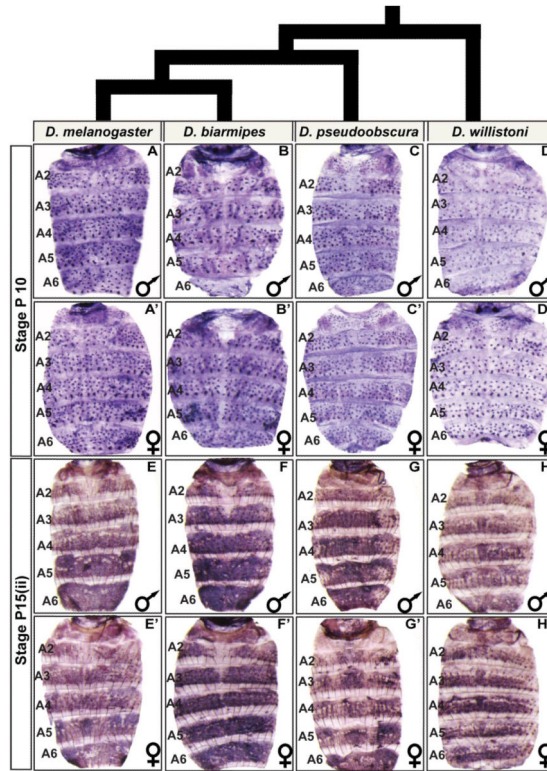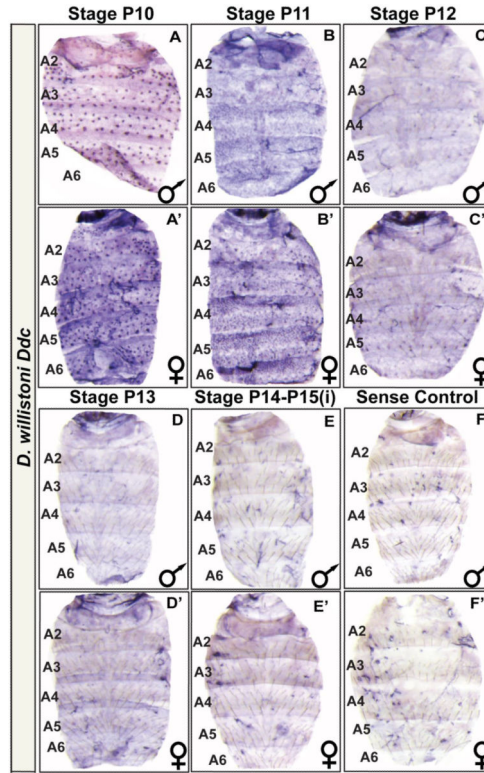
Fig. 3. *pale* abdominal epidermis expression is broadly conserved in *Sophophora*.
(A–H) *pale* expression in the dorsal abdomens of male and (A'–H') female pupae. At the
P10 stage, *pale* expression occurs robustly in the mechanosensory bristle cells of (A and A')
D. melanogaster, (B and B') *D. biarmipes*, (C and C') *D. pseudoobscura*, and (D and D') *D.
willistoni*. At the P15(ii) stage, *pale* expression is robust in the abdominal epidermis of (E
and E') *D. melanogaster*, (F and F') *D. biarmipes*, (G and G') *D. pseudoobscura*, and (H and
H') *D. willistoni*.
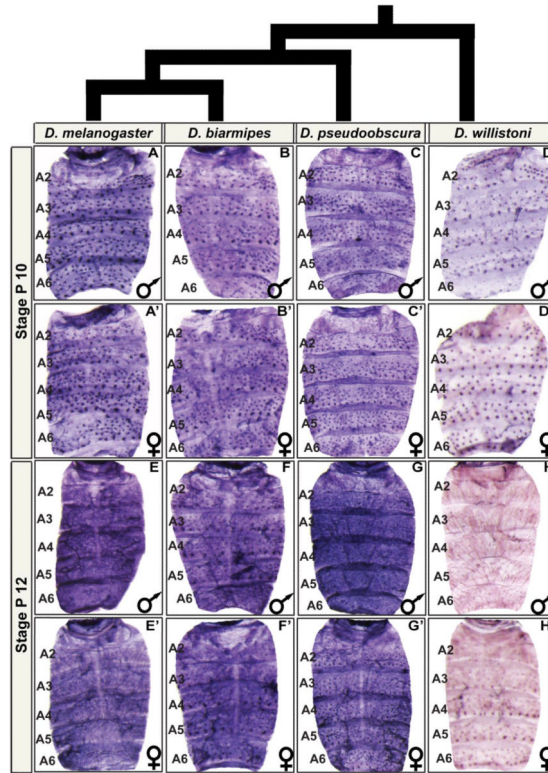
Fig. 4. ***Ddc* is broadly expressed in the abdominal epidermis of species with elaborate patterns of tergite pigmentation.**

Ddc expression in the dorsal abdomens of (A–H) male and (A'–H') female pupae. (A–D and A'–D') Expression at the P10 and (E–H and E'–H') P12 developmental stages. At P10, *Ddc* expression occurs robustly in the mechanosensory bristle cells of (A and A') *D. melanogaster*, (B and B') *D. biarmipes*, (C and C'), *D. pseudoobscura*, and (D and D') *D. willistoni*. At P12, expression has expanded to include the abdominal epidermis of (E and E') *D. melanogaster*, (F and F') *D. biarmipes*, and (G and G'), and *D. pseudoobscura*. (H and H') For *D. willistoni*, a similar robust pattern of *Ddc* expression was not observed at the P12 stage.

Fig. 5. *D. willistoni* exhibits little to no *Ddc* expression in the abdominal epidermis of various pupal stages through eclosion.

In situ hybridization for *Ddc* expression was performed on the dorsal abdominal epidermis of *D. willistoni* (A–F) male and (A'–F') female specimens. (A and A') *Ddc* is expressed in the mechansosensory bristle cells as early as the P10 stage of pupal development. (B and B') By the P11 stage, a low level of *Ddc* expression can be observed in the epidermis regions underlying where tergites will form. (C and C') By the P12 stage, little *Ddc* expression is observed in the epidermis regions underlying where tergites will form. (D and D') At the P13 and (E and E') P14-P15(i) stages, little to no expression was observed in the epidermis. (F and F') A *Ddc* sense probe control shows background signal observed in eclosed flies.
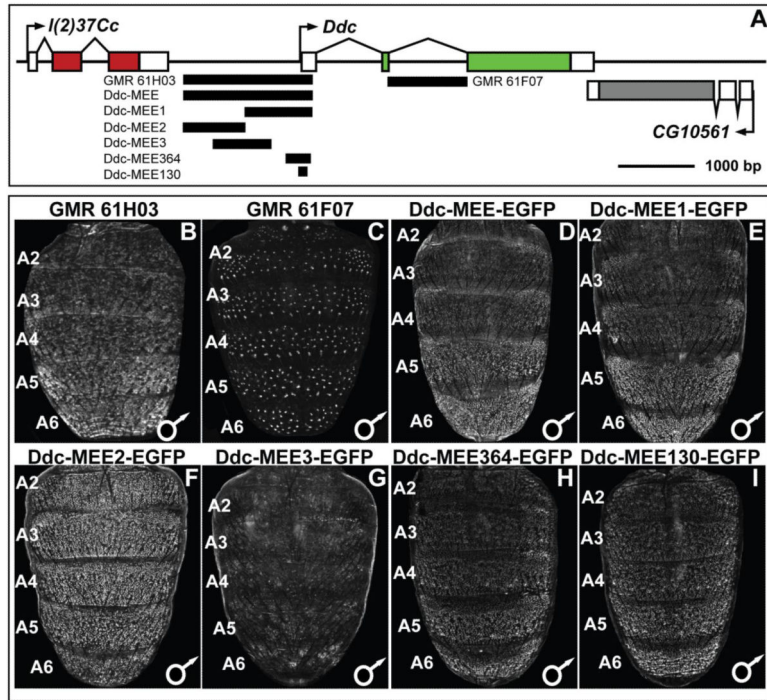
Fig. 6. An abdominal epidermis *cis*-regulatory element is located 5' of the endogenous *Ddc* promoter.

(A) To scale representation of the *Ddc* locus showing regions that were evaluated for CRE activity. (B–I) CRE activity observed by the expression of the EGFP reporter gene in the dorsal abdomens of P14-P15(i) stage transgenic male *D. melanogaster* pupae. (B and C) UAS-EGFP reporter expression driven by GAL4 expressed under the control of *Ddc* locus subregions. (B) The entire upstream region of *Ddc*, named the Ddc-MEE, drove reporter expression in the A2–A6 abdominal regions underlying where tergites develop, a pattern that mimics the endogenous *Ddc* expression. (C). The 2nd intron region of *Ddc* drove reporter expression in the mechanosensory bristle cells, a pattern that mimics the endogenous *Ddc* expression. (D–I) Expression of direct CRE-GFP fusion transgenes. (D) The entire *Ddc* upstream region drove reporter transgene expression in an abdominal epidermis patter similar to endogenous *Ddc* expression and that driven by the same region using the GAL4/UAS system. (E) The promoter-proximal Ddc-MEE1 subdivision of the Ddc-MEE retained the patterned abdominal epidermis activity. (F) The Ddc-MEE2 reporter drove expression more broadly in the abdominal epidermis than the Ddc-MEE CRE and endogenous *Ddc* expression. (G) The Ddc-MEE3 reporter lacks noteworthy abdominal activity. (H and I) Extreme truncated versions of the Ddc-MEE1 region to 364 and 130 base pairs had regulatory activities similar in pattern to that of the Ddc-MEE1 sequence.
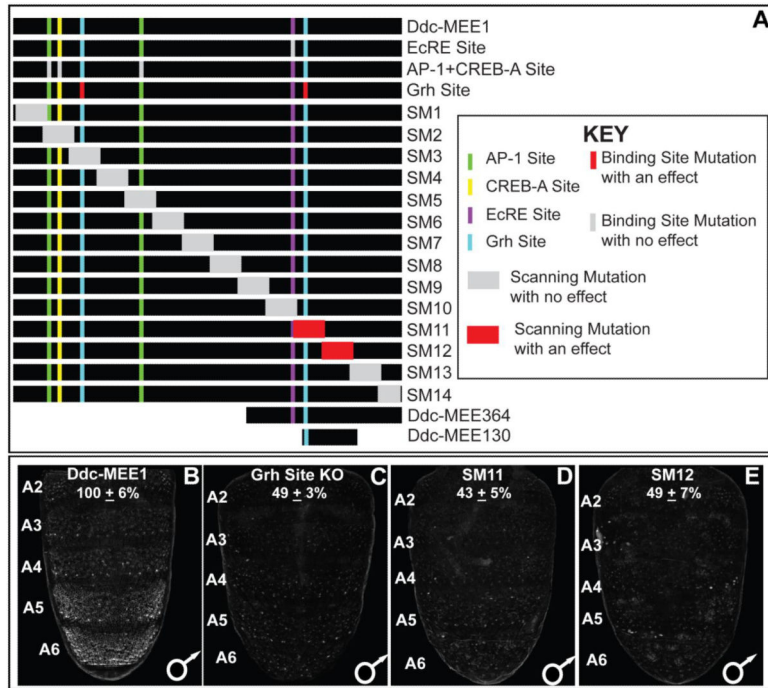
Fig. 7. A Grh binding site is a required input in the regulatory logic driving *Ddc* abdominal epidermis CRE activity.

(A) To scale annotation of the Ddc MEE1 region, and the mutant forms of the Ddc-MEE1 that were evaluated for alterations in regulatory activity. (B–E) Regulatory activities of CREs as seen by *EGFP* reporter gene expression in transgenic *D. melanogaster* abdomens. (B) Patterned CRE activity driven by the wild type Ddc-MEE1. (C) Compared to the wild type Ddc-MEE1, reporter activity was reduced to 49±3% when the two Grh binding sites were mutated. (D and E) Similarly, the scanning mutant 11 and 12 alterations respectively lowered Ddc-MEE1 reporter activity to 43±5% and 49±7% of the wild type element. Regulatory activity measurements are represented as the % of the wild type *D. melanogaster* Ddc-MEE1 mean A5 segment intensity plus the Standard Error of the Mean (SEM). Each activity measurement and SEM were derived using images for five biological replicates.
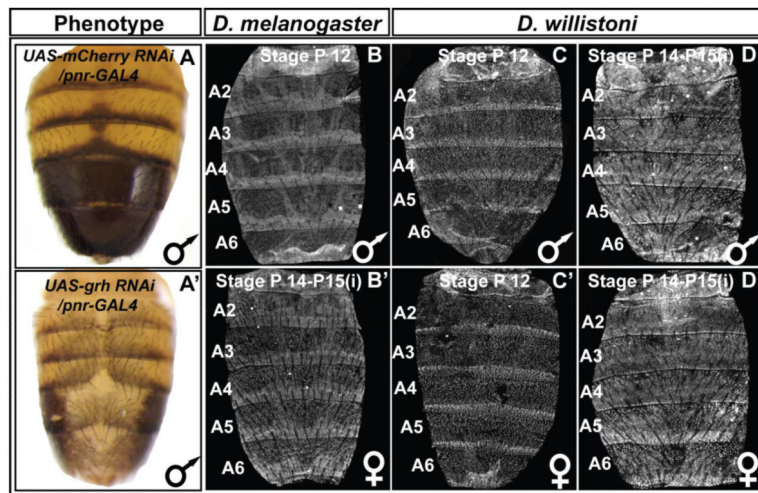
Fig. 8. Male-specific pigmentation requires the activity of Grh whose expression has remained conserved.

(A) A wild type pattern of pigmentation is exhibited in *D. melanogaster* males following RNA-interference, RNAi, for the negative control *mCherry* gene in the dorsal midline pattern of the *pnr* gene. (A') Melanic pigmentation was altogether lost in male abdomens following dorsal midline RNAi for the *grh* gene. Grh protein is expressed in the abdominal epidermis of (B) P12 and (B') P14-15(i) stage *D. melanogaster*. Similar patterns of Grh expression occur in (C and C') P12 and (D and D') P14-15(i) stage *D. willistoni*.
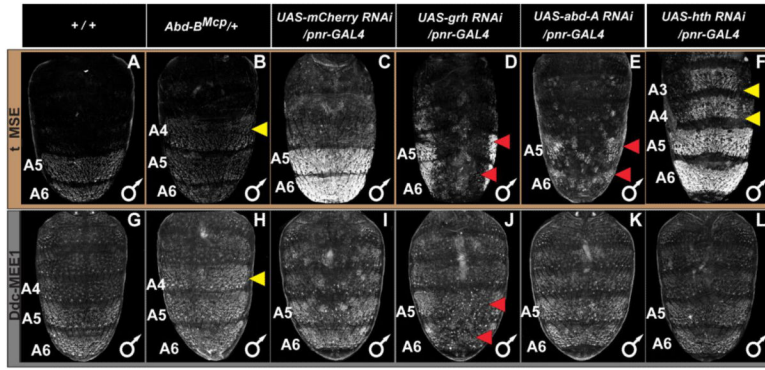
Fig. 9. Genetic interactions between pigmentation network transcription factors and CREs regulating *tan* and *Ddc* expression.

(A–F) EGFP reporter expression driven by the t_MSE was imaged at the P14-15(i) stage. (G–L) EGFP reporter expression driven by the Ddc-MEE1 was imaged at the P14-15(i) stage. The relevant genetic background genotypes are listed at the top of each column. All specimens are hemizygous for the *EGFP* reporter transgene. Compared to the (A and G) wild type genetic background, (B) t_MSE, and (H) Ddc-MEE1 regulatory activity expands into the A4 segment in which *Abd-B* is ectopically expressed. Compared to a (C) control genetic background, suppression of (D) *grh* expression resulted in a stark loss of t_MSE activity. In contrast, the suppression of (E) *abd-A* and (F) *hth* expression respectively led to reduced A5 and A6 segment t_MSE activity and an ectopic t_MSE activity in the A4 and A3 segments. Compared to Ddc-MEE1 activity in a (I) control genetic background, suppression of (J) *grh* expression resulted in a conspicuous loss of Ddc-MEE1 activity in the A5 and A6 segments, whereas suppression of (K) *abd-A* and (L) *hth* respectively had little to no effect on A5 and A6 segment Ddc-MEE1 activity, nor any ectopic activity in the A4 and A3 segments. Yellow arrowheads indicate segments where the genetic background alterations resulted in conspicuous ectopic EGFP reporter expression. Red arrowheads indicate segments where the genetic background alterations resulted in a reduced EGFP reporter expression.
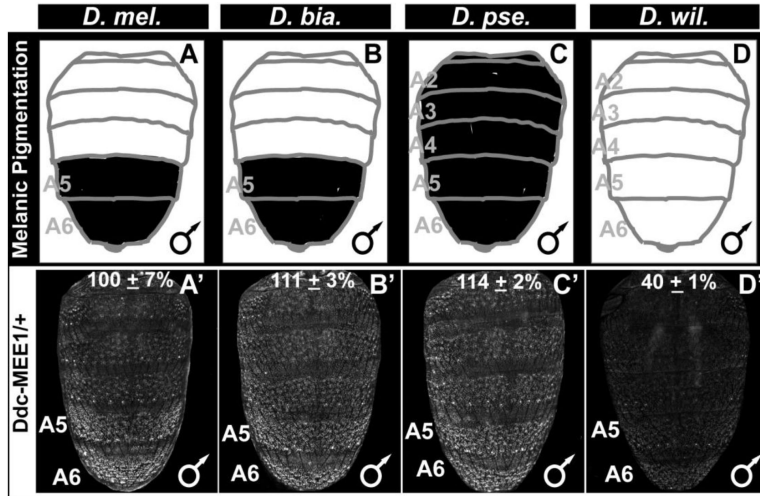
Fig. 10. The ancestry of the robust abdominal epidermis regulatory activity of the CRE controlling *Ddc* expression.
(A–D) A schematic representation of the male abdomen pigmentation patterns for several *Sophophora* species. (A'–D') EGFP-reporter transgene expression driven by sequences orthologous to the *D. melanogaster* Ddc-MEE1 in transgenic *D. melanogaster* male pupae at the P14-15(i) stage. (A'–C') Sequences from species with conspicuous melanic pigmentation phenotypes drive reporter expression throughout the abdomen, though the activity appears most pronounced in the A5 and A6 segments. (D') The sequence from the most distantly-related species with monomorphic tergite pigmentation, *D. willistoni*, has comparatively less abdominal regulatory activity. Regulatory activity measurements are represented as the % of the wild type *D. melanogaster* Ddc-MEE1 mean A5 segment intensity plus the Standard Error of the Mean (SEM). Each activity measurement and SEM were derived using images for five biological replicates.
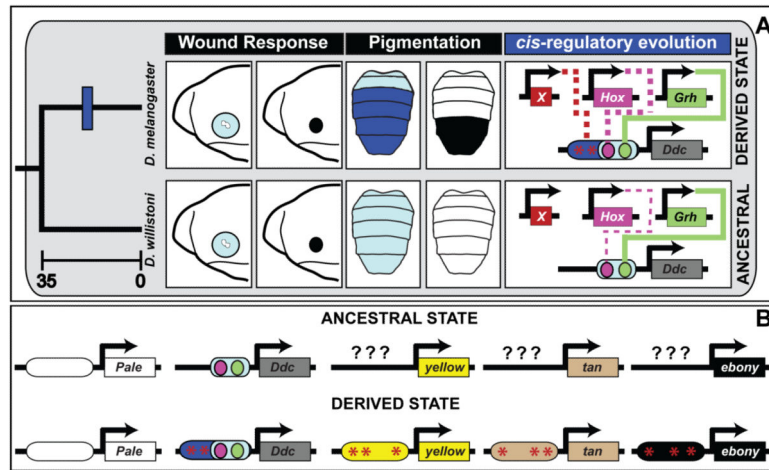
Fig. 11. The evolution of a novel pigmentation trait required *cis*-regulatory evolution at several realizator loci.

(A) In the embryo, *Ddc* expression (light blue) is upregulated around sites of epidermal wounding as part of a response that forms a melanized plug (black circle). Schematic depicts the anterior embryo. This gene function is conserved, and encoded in the activity of a CRE directly regulated by the Grh transcription factor. In *D. willistoni*, which serves as a proxy for the ancestrally monomorphic trait, *Ddc* expression is driven at a low level by the pleiotropic activity of the wound response CRE. This ancestral CRE possesses a direct binding site for Grh and is modestly-responsive to the Hox transcription factor Abd-B. In species with derived melanic abdomen pigmentation phenotypes, *Ddc* expression is robust throughout the abdomen (dark blue). This expression is in part due to *cis*-evolution at *Ddc*, which evolved a stronger, Hox-responsive CRE activity that is apparent for *D. melanogaster*, and this CRE seemingly evolved to be under the control of some *trans*-regulator(s) whose identity remains unknown and is represented here as factor "*X*". Time scale is depicted in millions of years. Solid lines in *cis*-regulatory evolution indicate cases where the transcription factor directly binds to the *Ddc* CRE, and dashed lines indicate cases where the mechanism of transcription factor regulation remains unknown. (B) Model for the origin of the derived dimorphic pigmentation trait of *D. melanogaster* from an ancestral monomorphic state. Here, abdominal *pale* expression was ancestral, whereas *Ddc*, *yellow*, *tan*, and *ebony* each evolved novel expression through changes in CREs controlling their expression. The ancestral states upon which novel CREs evolved for *yellow*, *tan*, and *ebony* remain unknown (question marks). For *Ddc*, *cis*-evolution augmented an ancestral wound response CRE to have a robust pleiotropic activity.