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The Regulation and Evolution of a Genetic Switch Controlling Sexually Dimorphic Traits in *Drosophila*

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SUMMARY

Sexually dimorphic traits play key roles in animal evolution and behavior. Little is known, however, about the mechanisms governing their development and evolution. One recently evolved dimorphic trait is the male-specific abdominal pigmentation of *Drosophila melanogaster*, which is repressed in females by the Bric-à-brac (Bab) proteins. To understand the regulation and origin of this trait, we have identified and traced the evolution of the genetic switch controlling dimorphic *bab* expression. We show that the HOX protein Abdominal-B (ABD-B) and the sex-specific isoforms of Doublesex (DSX) directly regulate a *bab* cis-regulatory element (CRE). In females, ABD-B and DSX^F activate *bab* expression whereas in males DSX^M directly represses *bab*, which allows for pigmentation. A new domain of dimorphic *bab* expression evolved through multiple fine-scale changes within this CRE, whose ancestral role was to regulate other dimorphic features. These findings reveal how new dimorphic characters can emerge from genetic networks regulating pre-existing dimorphic traits.

INTRODUCTION

Sexual dimorphism is widespread in the animal kingdom. Males and females differ in primary reproductive structures and are often distinguished by secondary sexual characteristics, some of which have evolved through sexual selection (Darwin, 1871). Some of the most conspicuous modifications of these secondary traits involve male morphology, such as the lion's mane and the peacock's elaborate tail. While the ecological roles of sexually dimorphic traits have been well-studied (Andersson, 1994), relatively little is known about how specific traits are produced or have evolved (Wilkins, 2004).

The development of a trait in one sex and not the other must be the result of differential gene expression. Correlations have been found between gene expression patterns and dimorphic trait production. For example, *msxC* expression is associated with the development of the male "sword" of the swordtail fish (Zauner et al., 2003) and the male-specific pattern of *Sex combs reduced* (*Scr*) correlates with the divergence in sex comb formation, size, and morphology among *Drosophila* species (Barmina and Kopp, 2007). However, the regulatory mechanisms governing these gene expression patterns or the development of any male-limited morphological trait have not been elucidated.

While in vertebrates, sex-specific gene expression is often mediated by sex-specific hormones and their receptors (Robins, 2005; Verrijdt et al., 2003), in *C. elegans* and *D. melanogaster*, sex-specific gene expression is mediated by the transcriptional effectors of their sex-determination pathways. In these species, homologous regulatory proteins of their somatic sex-determination pathways bind to CREs for the *yolk protein* (*yp*) genes and regulate their female-specific expression (An and Wensink, 1995; Coschigano and Wensink, 1993; Yi et al., 2000; Yi and Zarkower, 1999).

Because of the prominent roles sexually dimorphic traits play in animal behavior and evolution, the origins of sex-limited traits has been of special interest in evolutionary biology. From the standpoint of developmental genetics, the central question has been: Do new traits and gene expression patterns arise by the gain of expression of genes in one sex that were not previously expressed in either sex or, alternatively, does dimorphism evolve by the repression of traits and genes in one sex that were formerly expressed in both sexes?

One promising model for understanding the development and evolution of dimorphic traits is the recently evolved male-specific pigmentation of the *melanogaster* species group in the genus *Drosophila*. Males of this clade typically have fully pigmented dorsal cuticular plates (tergites) on abdominal segments A5 and A6 (Figure 1A). In females (Figure 1B), A5 and A6 tergite pigmentation is restricted to a posterior stripe, similar to that present on the tergites of abdominal segments A2–A4 of both sexes. Much is known about the developmental genetics of

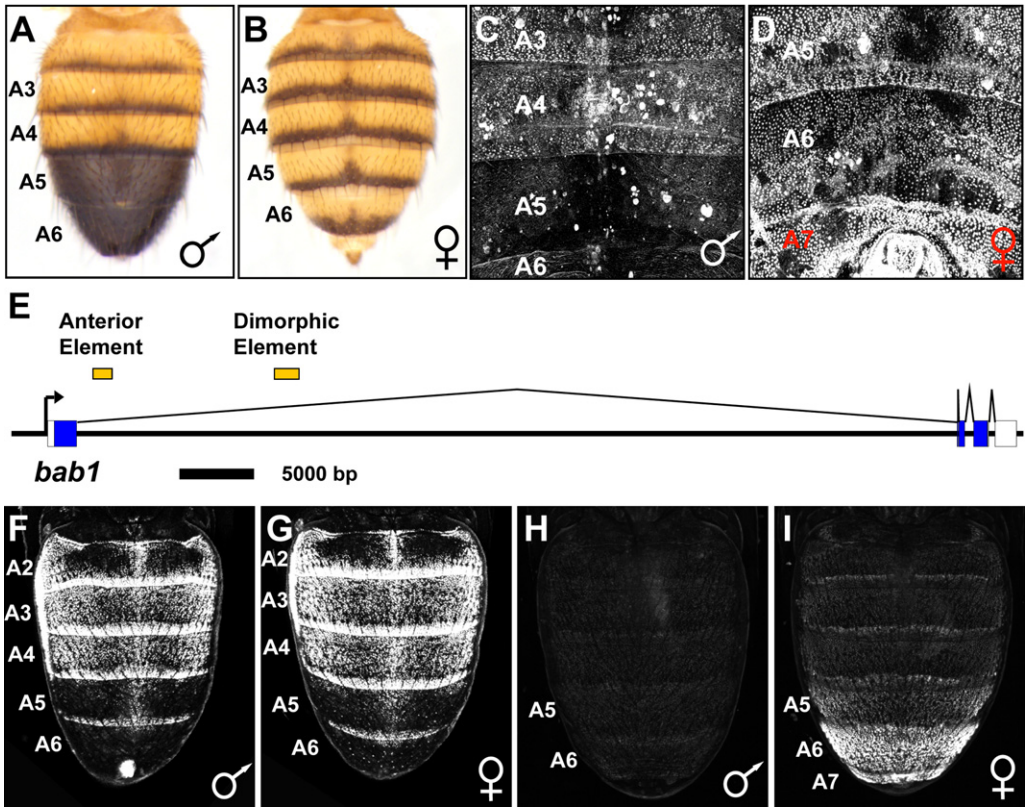

Figure 1. Bab1 Expression in the Abdomen Is Regulated by Two CREs

(A and B) Dorsal view of *D. melanogaster* adult abdomens. Male segments A5 and A6 are fully pigmented (A). In females, pigmentation of these segments is limited to a posterior stripe (B).

(C and D) Expression of Bab1 in male and female pupae at 72 hr APF. Bab1 expression in males is limited to segments A2–A4 (C), but in females, Bab1 expression extends into segments A5 and A6, as well in the female-specific segment A7 (D).

(E) Two CREs, the anterior element and dimorphic element, reside in the large 1st intron of *bab1* and govern Bab expression in the abdominal epidermis.

(F–I) GFP-reporter expression in dorsal pupal abdomens.

(F and G) The anterior element drove GFP-reporter gene activity in segments A2-A4 of both males (F) and females (G).

(H) The dimorphic element was inactive in males.

(I) The dimorphic element drove reporter expression in female segments A5–A7, with levels increasing from the anterior to posterior.

this pattern in *D. melanogaster*, which requires the activities of several enzymes involved in pigment production as well as several transcription factors (Wittkopp et al., 2003). Two central regulators of posterior pigmentation are the proteins encoded by the tandemly duplicated genes *bab1* and *bab2* of the *bab* locus. Both genes encode DNA-binding proteins (Lours et al., 2003) that act as dominant repressors of pigmentation (Couderc et al., 2002; Kopp et al., 2000). While female pupae express *bab* in abdominal segments A2–A6, *bab* expression in males is limited to segments A2–A4, and the relative absence of *bab* expression in segments A5 and A6 is necessary for their greater pigmentation in males (Kopp et al., 2000). Genetic analyses have implicated the *Hox* gene *Abd-B* as a repressor of *bab* in these posterior segments and suggested that repression of *bab* is mitigated in females by the activity of dsx^F, the sex-specific transcript derived from the *doublesex* (*dsx*) locus (Kopp et al., 2000). The male-specific repression of *bab* appears to have evolved from an ancestral monomorphic condition in which *bab* was expressed in the posterior of both sexes.

In order to understand the molecular mechanisms by which *bab* expression is regulated and has evolved, we sought to identify the CREs governing *bab* expression, to characterize the direct transcriptional regulators of their CREs, and to trace how functional changes in gene expression have occurred in *Drosophila* evolution. We found that two CREs govern *bab* expression in the pupal abdomen. These include one element that regulates *bab* expression in segments A2–A4 of both sexes and a second, dimorphic element that regulates expression in the posterior segments A5–A7 of females. We demonstrate that the dimorphic element is part of a genetic switch that, in combination with the HOX protein ABD-B and the sex-specific activities of the male and female isoforms of the DSX protein, directs female-specific activation and male-specific repression of *bab* in posterior segments. Surprisingly, we found that both the presence of this dimorphic CRE and its regulation by ABD-B and DSX predated the origin of dimorphic pigmentation. We discovered that the new domain of dimorphic CRE activity required for dimorphic pigmentation evolved from many fine-scale

changes within the CRE. Our results show how new dimorphic characters can evolve from the genetic regulatory architecture governing other dimorphic traits.

RESULTS

Bab1 Is Expressed in a Dimorphic Pattern

Genetic studies have shown that both *bab1* and *bab2* are required for the development of the wild-type *D. mel*. pigmentation pattern (Couderc et al., 2002; Kopp et al., 2000), and previous work has shown that Bab2 is expressed dimorphically in the pupal abdominal epidermis (Gompel and Carroll, 2003; Kopp et al., 2000). However, the expression of Bab1 has not been fully characterized and it was important to determine whether both proteins were expressed and therefore regulated in similar ways. In order to analyze the expression of Bab1, we developed an affinity-purified polyclonal antibody specific to Bab1 that did not cross-react with Bab2. We determined that during pupal development Bab1 was also expressed in segments A2–A4 of males with the highest levels occurring in A3 and A4 (Figure 1C), whereas in females Bab1 is expressed in segments A2 through A7 (Figure 1D). Therefore, both Bab1 and Bab2 are expressed dimorphically in the abdominal epidermis.

Two *cis*-Regulatory Elements Direct Bab Expression in the Abdomen

In order to dissect the molecular mechanisms regulating dimorphic *bab* expression, we sought to identify the CREs within the *bab* locus that govern gene regulation in the abdomen. We conducted a systematic screen of the ~150 kb of noncoding sequence between the neighboring *trio* and *CG13912* loci, excluding transposon-derived sequences, for regions with regulatory activity. Overlapping segments of DNA with a typical size of 7 kb were tested for their ability to direct reporter gene expression in the abdomens of transgenic *D. mel.* pupae (Figure S1A available online). Consistent with the pleiotropic roles of the Bab transcription factors (Couderc et al., 2002), we identified several separate CREs that drove reporter expression in specific tissues during pupal development, including the oenocytes, legs, bristles, and abdominal muscles (Figure S1).

Most importantly, two separate CREs were identified in the large first intron of *bab1* that drove reporter expression in the pupal abdominal epidermis (Figures 1E and S1). One CRE, which we refer to hereafter as the "anterior" element (1357 base pairs (bp)), drove reporter expression in a monomorphic pattern in segments A2–A5 of both sexes with significantly lower levels of expression in segments A2 and A5 than in segments A3 and A4 (Figure 1F and 1G). The second CRE, referred to as the "dimorphic" element (663 bp), drove a female-specific pattern of reporter expression in segments A5–A7 (Figure 1H and 1I). A construct containing both elements drove reporter gene expression in a spatial pattern similar to the endogenous expression of Bab1 and Bab2 in the epidermis of the abdomen (Figures S1F and S1G). Since the regulatory elements are located in the first intron of *bab1*, we conclude that these CREs regulate *bab1 in vivo*. Furthermore, since no other CREs were found in the entire locus that directed gene expression in the abdomen, and *bab2* is expressed in a similar pattern, we infer that the CREs also regulate *bab2* expression.

Regulation of Bab Expression by *Abd-B* and *Dsx*

The segment- and sex-specific activity of the *bab* CREs suggested that they are regulated by segment- and sex-specific transcription factors. In order to elucidate the factors regulating these CREs, we examined the activity of the anterior and dimorphic elements in mutant genetic backgrounds. The best candidate segment-specific regulator is the product of the *Hox* gene *Abdominal-B* (*Abd-B*), which is expressed at increasing levels from pupal abdominal segment A5 to A7 (Kopp and Duncan, 2002). Furthermore, *Abd-B* behaves genetically as a repressor of pigmentation and *bab* expression (Kopp et al., 2000). Consistent with these observations, compared to activity in a wild-type genetic background (Figure 2A), reporter gene expression driven by the anterior element was repressed in both males (Figures 2B and 2C) and females (data not shown) when *Abd-B* was ectopically expressed in segments anterior to A5. Conversely, the activity of the dimorphic element was expanded by ectopic expression of *Abd-B* in females (Figures 2D–2F). Ectopic expression of *Abd-B* had no effect on dimorphic element activity in males (data not shown). Thus, *Abd-B* behaves genetically as an activator of the dimorphic element and as a repressor of the anterior element.

However, genetic regulation by *Abd-B* cannot account for the absence of dimorphic element activity in males, as *Abd-B* expression is monomorphic. One candidate for controlling sex-specific gene expression is the *doublesex* (*dsx*) gene. dsx^{-} flies exhibit an intermediate sexual identity (intersex) and their pigmentation on segments A5 and A6 is similar to that of a wild-type male (Jursnich and Burtis, 1993; Kopp et al., 2000). Transcripts from the *dsx* locus are alternatively spliced between males and females (Burtis and Baker, 1989), and these alternative transcripts encode sex-specific protein isoforms (DSX^{F} and DSX^{M}) which share a common N-terminal DNA-binding domain but have variant C-terminal domains that mediate different cofactor interactions (Garrett-Engele et al., 2002). To test whether dsx^{M} genetically represses dimorphic element activity, we compared reporter activity in a chromosomal female genetic background ($dsx^{D/+}$) where the transcript produced by one allele of *dsx* is spliced as dsx^{M}. In these intersexual pupae, reporter activity in segments A6 and A7 was reduced to 68% and 36%, respectively (Figures 2I and 2L) of the activity in a wild-type background (Figures 2G and 2J). This reduction was due to the gain of dsx^{M} activity rather than haplo-insufficieny for dsx^{F}, because reporter activity in a female $dsx^{+/-}$ background (Figures 2H and 2K) was equivalent to that measured in the wild-type background (Figures 2G and 2J). These results demonstrate that sex-specific regulation of the dimorphic element is provided by dsx^{M} acting as a repressor in males, and most likely by dsx^{F} acting as an activator in females. In order to understand the molecular mechanisms of the dimorphic regulation of *bab* and how it evolved, we next sought to identify which of these regulatory interactions were direct.

ABD-B Is a Direct Segment-Specific Activator of the Dimorphic Element

In order to determine if ABD-B is a direct regulator of *bab* CREs, we examined the anterior and dimorphic element for ABD-B

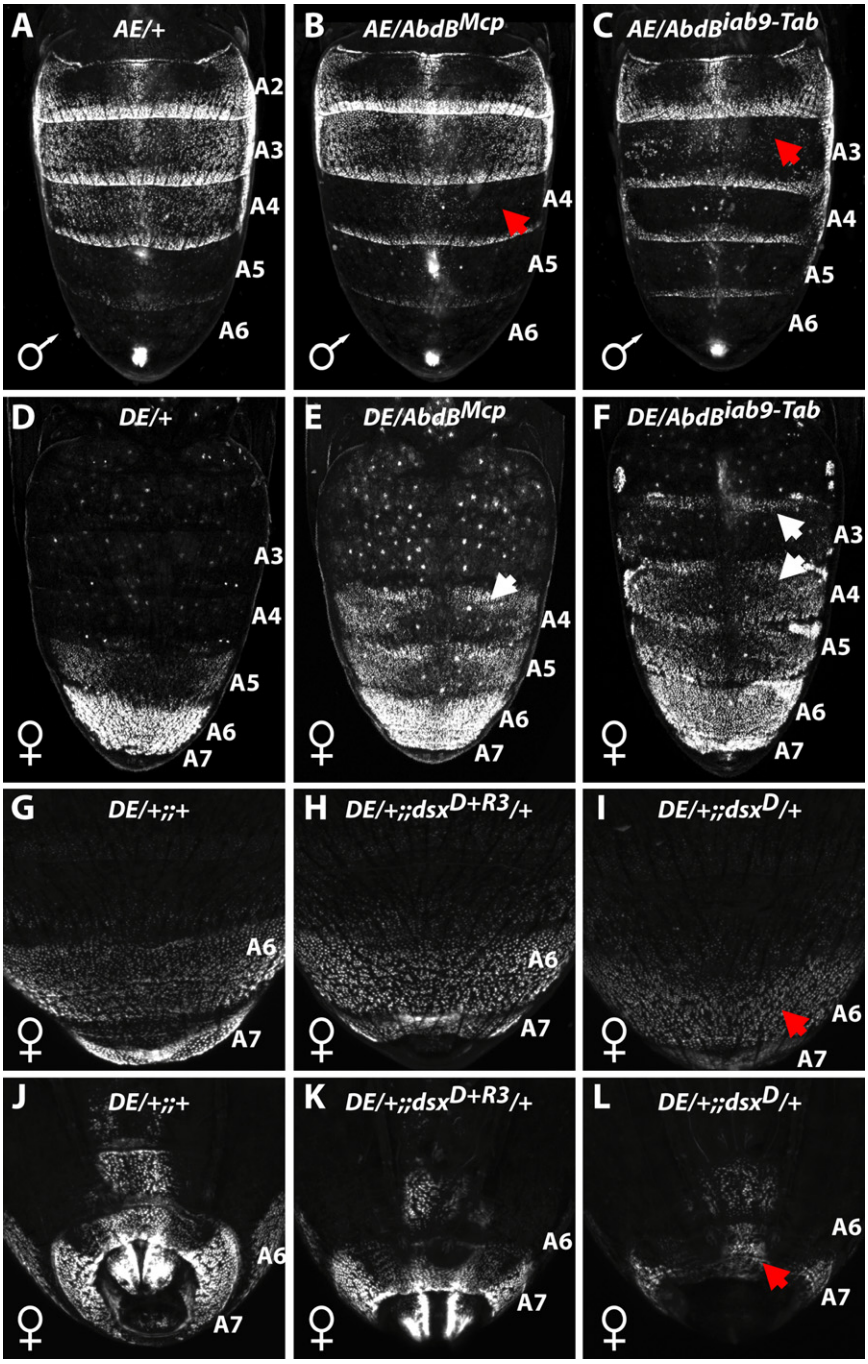

Figure 2. Genetic Regulation of *bab* CRE Activity by the *Abd-B* and *Dsx* Loci

Images are of dorsal (A–I) and ventral (J–L) abdomens of pupae heterozygous for GFP-reporter constructs. Genotypes are listed at the top of each image. The anterior element is denoted as "*AE*" and the dimorphic element "*DE*." Specimens are heterozygous for the $AbdB^{Mcp}$ (B and E), $AbdB^{iab9\text{-}Tab}$ (C and F), dsx^{D+R3} (H and K), and dsx^{D} (I and L) mutant allele. Red and white arrows indicate regions where reporter activity in the mutant background is decreased or increased, respectively, compared to the wild-type control.

(A) The *AE* drove reporter expression in the anterior segments A2–A4.

(B and C) Ectopic expression of *Abd-B* in segments A4 (B) and A3–A4 (C) resulted in repression of *AE* activity in these segments.

(D, G, and J) The *DE* drove reporter expression in the posterior segments A5–A7 of females.

(E and F) Ectopic expression of *Abd-B* in segments A4 (E) and A3–A4 (F) resulted in ectopic *DE* activity in these segments.

(H and K) In a *dsx* heterozygous null mutant genetic background, *DE* activity is indistinguishable from that in a wild-type background.

(I and L) In a chromosomal female intersex, where one *dsx* allele is producing the male transcript instead of the female, *DE* activity was reduced to 68 in A6 (I) and to 36% in A7 (L) of the activity of the *DE* in wild-type females.

binding sites. ABD-B and paralogous HOX proteins have been shown to preferentially bind to the five base pair motifs TTTAT or TTTAC, and with some affinity to the shorter motif TTAT (Ekker et al., 1994). Within the dimorphic element there were, remarkably, fourteen sites with the core sequence TTTAT or TTTAC (Figure 3A) and eight additional TTAT sites. DNaseI footprinting with purified ABD-B homeodomain (HD) protein identified that most of these sequences were bound (12 of the 14 core sites; Figure S2). To determine whether CRE activity required the full 663 bp of the dimorphic element containing all of these sites, truncation constructs that included subsets of the candidate ABD-B sites were tested for reporter activity. Compared to the wild-type element (Figure 3B), the truncated elements exhibited dramatic decreases in (Figures 3C and 3E) or the elimination (Figure 3D) of CRE activity. These results indicate that sequences required for full CRE activity are distributed throughout the entire dimorphic element.

In order to determine whether the candidate ABD-B sites were required for CRE activity in vivo, these sequences were mutated alone or in combination (from TTAT/TTAC to CGGC) within the context of the 663 bp dimorphic element, and the effects on reporter activity were analyzed in transgenic female pupae. In order to eliminate the potential variation introduced by the site of transgene insertion, comparisons between wild-type and mutant elements were performed with transgenes inserted into the same chromosomal position. We consistently observed low inter- and intra-transgenic line variation in reporter activity, which enabled us to detect modest quantitative changes in reporter activity.

Mutation of all TTAT sequences (a total of 15 sites) reduced reporter activity to 9 ± 0% (%activity ± SEM) of the wild-type dimorphic element activity in segment A6 and substantially

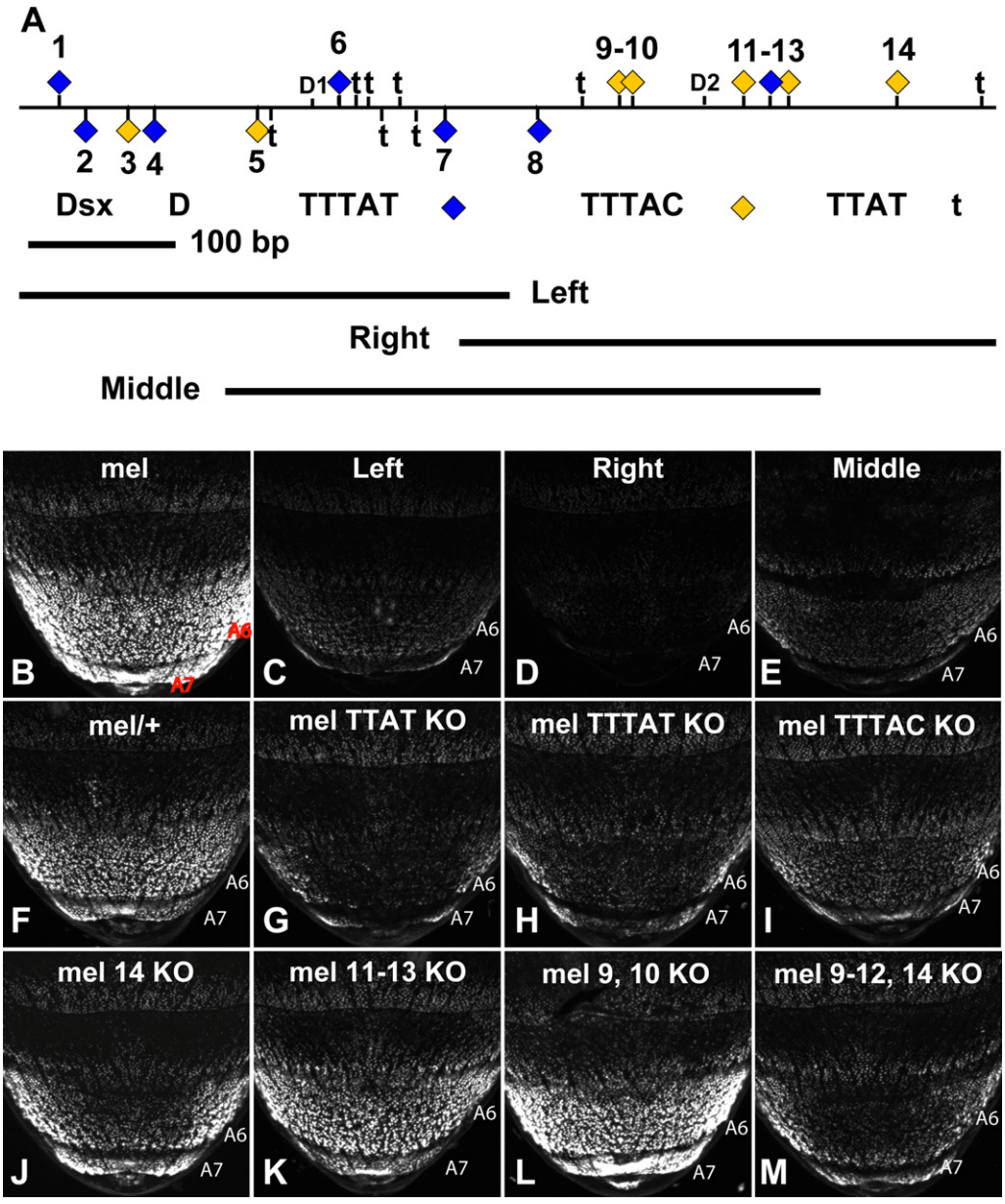

Figure 3. The Dimorphic Element Is Directly Regulated by ABD-B through Multiple Binding Sites

(A) Schematic of the minimal *D. mel.* wild-type (mel) CRE sequence conferring robust female-specific activity, with the location of putative ABD-B (yellow and blue diamonds) and DSX (D1 and D2) binding sites indicated. Additional TTAT (non-TTTAT) motifs are indicated by "t." The spans of the Left, Right, and Middle subconstructs are indicated below the schematic.

(B–M) Comparison of GFP-reporter gene activity in transgenic female pupae at 75 hr APF. Activity measurements are represented as the % of the wild-type (mel) female A6 mean ± SEM.

(B) The wild-type dimorphic element drove reporter expression at high levels in A6 and A7.

(C–E) Truncation of the dimorphic element into Left (C), Right (D) and Middle (E) sub-fragments resulted in dramatically decreased reporter activity.

(F) Reporter activity is reduced to 59 ± 2% in pupae heterozygous for the dimorphic element reporter transgene.

(G–L) Activity of dimorphic elements in which subsets of putative ABD-B binding sites have been mutated.

(G) Mutation of all fifteen TTAT sites reduced reporter activity to 9 ± 0%.

(H) Mutation of all seven TTTAT sites reduced reporter activity to 19 ± 2%.

(I) Mutation of all seven TTTAC sites reduced reporter activity to 26 ± 3%.

(J) Mutation of ABD-B site 14 reduced reporter activity to 55 ± 0%.

(K) Mutation of ABD-B sites 11-13 reduced reporter activity to 79 ± 4%.

(L) Mutation of ABD-B sites 9 and 10 had no detectable affect on dimorphic activity.

(M) Mutation of ABD-B sites 9-12 and 14 reduced reporter activity to 26 ± 6%.

reduced reporter expression in segment A7 (Figure 3G). Mutation of the seven TTTAT sites reduced activity to 19 ± 2% of the wild-type element (Figure 3H). This slightly lesser reduction in the TTTAT site mutant than in the TTAT site mutant indicated that there was some contribution of the TTAT sites to full CRE activity. Mutation of all seven TTTAC sites reduced activity to 26 ± 3% of wild-type activity (Figure 3I), further indicating that regulation of the dimorphic element by ABD-B in vivo is mediated through many binding sites.

In order to better understand the contribution of individual or small groups of ABD-B binding sites to CRE activation, we mutated putative sites individually and in combination and analyzed their effects on reporter activity. Mutation of one or few sites ranged between having a large (Figure 3J), modest (Figure 3K, and Figure 6B) or no measurable effect (Figures 3L and 6C) on CRE activity. When more binding sites were mutated in combination, CRE activity was reduced further (Figure 3M). Collectively, these results demonstrate that ABD-B mediates the segment-specific activation of the dimorphic element by the cumulative effects of binding to many sites.

In contrast to the dimorphic element, a similar mutational analysis of the *bab* anterior element provided no evidence for its direct regulation by ABD-B. We conclude that the repression of the anterior element in segments A5 and A6 is mediated indirectly by *Abd-B* through regulation of other genes. We have therefore focused our subsequent analyses almost exclusively on the regulation and evolution of the dimorphic element.

Sex-Specific Isoforms of DSX Directly Regulate Female-Specific Activation and Male-Specific Repression of the Dimorphic Element

To determine whether the DSX proteins were direct sex-specific regulators of the dimorphic element, DNase I footprinting was performed with the DSX DNA-binding domain (DBD) on the minimal sequence sufficient for full dimorphic activity. Two sites were identified within the dimorphic element that were the only matches to an empirically determined consensus sequence for DSX binding (Erdman et al., 1996). The first site (Dsx1; Figure 4A), resides between ABD-B site 5 and 6 and the second site (Dsx2; Figure 4B), lies between ABD-B sites 10 and 11 (Figure 3A). DSX binding to these two sites was specific (Figures 4C and 4D, lanes 1–4), as mutations in them significantly decreased binding by DSX (Figures 4C and 4D, lanes 5–8).

To resolve whether DSX binding to sites Dsx1 and Dsx2 was required for dimorphic element function in vivo, GFP-reporter expression was monitored in transgenic pupae in which one or both of these sites were mutated. The wild-type CRE drove strong expression in segments A6 and A7 of female pupae (Figure 4E) and expression in males was limited to a low level in segment A6 (Figure 4I). When the Dsx1 site was mutated, reporter activity in females was reduced to 23 ± 2% of the wild-type element (Figure 4F). Similarly, when the Dsx2 site was mutated, reporter activity was reduced to 34 ± 3% (Figure 4G). However, neither mutation of the Dsx1 nor of the Dsx2 site alone altered the low level of reporter activity in males (Figure 4J and 4K). When both the Dsx1 and Dsx2 sites were mutated in combination, reporter activity in females was reduced to 24 ± 1% of the wild-type element (Figure 4H), and the reporter activity in male pupae was increased to 53 ± 3% to that of the wild-type dimorphic element in female pupae (Figure 4L).

These data demonstrate that the dimorphic element is directly regulated by the sex-specific isoforms of DSX and that, together with ABD-B, the two regulators and the CRE act as a segment- and sex-specific genetic switch to differentially regulate *bab* expression in the posterior segments of males and females. DSX^F acts in conjunction with ABD-B to activate the CRE in females, whereas the DSX^M isoform directly represses activity in males and overrides the positive regulation by ABD-B.

The recent evolution of dimorphic pigmentation and the operation of this genetic switch for controlling *bab* expression raises the question of how the switch evolved. Is the dimorphic element novel to *D. melanogaster* lineage? When did binding sites for and regulation by ABD-B and DSX evolve? In order to address these questions, we traced the evolutionary history of the *bab* CREs in the *Drosophila* genus.

The Dimorphic Element Has a Deep Ancestry

Monomorphic abdominal pigmentation (Jeong et al., 2006) and monomorphic Bab2 expression (Gompel and Carroll, 2003; Kopp et al., 2000) have been inferred to be the ancestral states within the subgenus *Sophohora* from which male-specific dimorphic pigmentation evolved in the *D. mel.* lineage. In order to trace the evolution of dimorphic *bab* expression, we selected *D. willistoni* (*D. wil.*) as a member of the monomorphic outgroup to the melanogaster species group and as a surrogate of the inferred ancestral monomorphic pigmentation state. In this species, pigmentation of segments A5 and A6 in males is limited to a posterior stripe, like that of females (Figures 5A and 5E). We found that Bab1 was expressed in a monomorphic pattern in segments A5 and A6 of *D. wil.* males (Figures 5B and 5C) and females (Figures 5F and 5G), similar to that shown for Bab2 (Kopp et al., 2000). Hence, the evolution of dimorphic pigmentation in the *melanogaster* species group involved the evolution of the repression of both Bab1 and Bab2 in the posterior segments of males.

The evolutionary transition from monomorphic to dimorphic *bab* expression could have occurred through a variety of mechanisms including the evolution of new *bab* CREs, modifications to orthologous CREs that altered their regulatory function, or changes in the deployment or activity of transcription factors that regulate *bab*. Since *dsx* function and expression (Cho et al., 2007; Hediger et al., 2004) and *Abd-B* expression (Yoder and Carroll, 2006) are well-conserved in the family Drosophilidae, we focused on the evolution of *bab* CREs. It was necessary to first identify *D. wil. bab* CREs that drove gene expression in the abdomen. We analyzed the orthologous sequences in the first intron of the *D. wil. bab1* gene for CREs with activity in transgenic *D. mel.* pupae. We identified a large region (5.8 kb) that contained orthologous sequence to the anterior element of *D. mel.*, and that drove GFP-reporter expression in the abdominal epidermis in segments A2 through the posterior-most segment in both sexes (Figures 5D and 5H), patterns similar to those of the endogenous Bab1 protein in *D. wil*. Thus, in the evolution of the *D. mel.* lineage the function of the anterior element was modified such that it no longer drove *bab* expression in segments A5 and A6 of either sex.

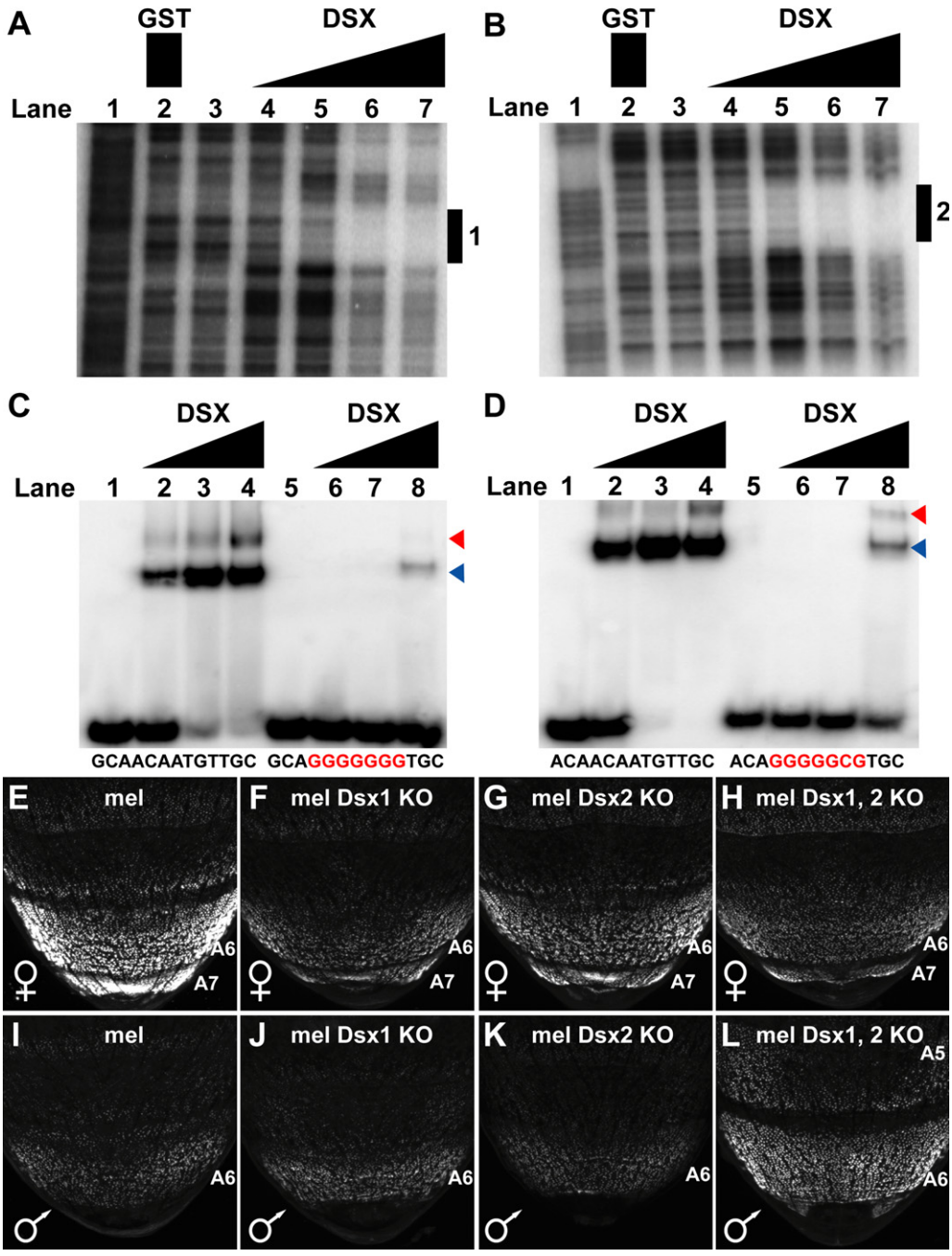

Figure 4. The Dimorphic Element Is Directly Regulated by Sex-Specific Isoforms of DSX

(A and B) DNaseI footprinting analysis of the dimorphic element with a GST-DSX DBD fusion protein identified two distinct sites bound by the DSX DNA-binding domain, referred to as Dsx1 (A) and Dsx2 (B). Amounts of each protein used were as follows: lane 2, 1,000 ng GST only; lane 3, no protein; lane 4, 64 ng DSX DBD; lane 5, 160 ng DSX DBD; lane 6, 400 ng DSX DBD; lane 7, 1,000 ng DSX DBD. A G+A sequencing ladder is included in lane 1. Footprinted regions are indicated by a black rectangle with an adjacent number.

(C and D) EMSAs on annealed oligonucleotide probes containing wild-type (lanes 1–4) and mutant (lanes 5–8) DSX binding sites. Below are the sequences of the wild-type and mutant Dsx1 (C) and Dsx2 (D) binding sites with mutated bases shown in red. For each probe, binding reactions were performed using increasing amounts of the DSX DBD protein (from left to right: 0 ng, 16 ng, 62 ng, 250 ng, and 1000 ng). Blue and red arrowheads point to the respective locations on the gel of complexes containing a single or pair of DSX DBD monomers bound to the probe.

(E–L) GFP-reporter activity in pupae at 75 hr APF. Activity measurements are represented as the % of the wild-type (mel) female A6 mean ± SEM.

(E and I) Activity of the wild-type dimorphic element was much greater in the female (E) than the male (I).

(F and J) When the Dsx1 site was mutated, activity in the female was reduced to 23 ± 2% (F), while activity in the male was unchanged (J).

The activity of the *D. wil.* anterior element in males and females would appear sufficient to account for monomorphic *bab* expression and hence monomorphic pigmentation. This observation raised the possibility that the dimorphic element was novel to the *D. mel.* lineage. However, we also identified a *D. wil.* sequence orthologous to the dimorphic element, which drove reporter expression in *D. mel.* female segment A7, but not in segments A5 and A6 (Figures 5I–5L). These findings suggested that an active dimorphic element existed in a common ancestor of *D. wil.* and *D. melanogaster*. To test this idea further, we also isolated an orthologous dimorphic element from the more distantly related species *D. virilis*. The activity of this element was also limited to the female segment A7 (data not shown).

These results demonstrate that the dimorphic element of *D. mel.* has a deep ancestry and did not arise de novo in the *D. melanogaster* lineage. Rather, the CRE existed in a common ancestor of monomorphically and dimorphically pigmented species. In *D. mel.*, *bab* is required to shape the development of particular features of the female-specific A7 segment (this segment is greatly reduced in males) (Kopp et al., 2000) and this is likely to be a deeply conserved role. Therefore, the *D. mel.* pattern of *bab* expression in segments A6 and A5 of females represents an expansion in the activity of the dimorphic element and the evolution of dimorphic pigmentation involved the functional modification of both ancestral *bab* CREs. We next sought to identify the sequence differences between the orthologous dimorphic elements that were responsible for their different activities.

Remodeling of the Dimorphic Element Underlies the Evolution of Expanded Dimorphic Bab Expression

Since the *D. mel.* dimorphic element had fourteen putative ABD-B binding sites, one possible explanation for the expanded activity of the *D. mel.* dimorphic element would be the evolutionary gain of ABD-B binding sites and, hence, ABD-B regulation throughout segments A5 and A6. However, we were surprised to find that the *D. wil.* element contained fourteen ABD-B binding sites. Alignment of the orthologous dimorphic elements' DNA sequences (Figure S5) revealed that the *D. wil.* element was collinear with the *D. mel* element and contained twelve of the fourteen *D. mel.* sites, and lacked only sites 8 and 13. The pair-wise differences between the two species could be due to either gains in the *D. mel.* lineage or losses in the *D. wil.* lineage, or both. To assess which was the case, we examined other taxa for the presence or absence of these ABD-B sites (Figure S5). Binding site 8 was identified in dimorphic elements from more distantly related non-Sophohoran species, indicating that this site was lost in the *D. wil.* lineage. The *D. mel.* site 13, however, was inferred to be a gain within the *melanogaster* species group, as this site did not occur in species outside of this clade, including representatives of the *obscura* and *saltans* groups. Conversely, the *D. mel.* element lacked two TTTAT sites that were adjacent to *D. wil.* site 12 that have been acquired since its divergence from the last common ancestor shared with *D. melanogaster.*

Although there is no net difference in the number of ABD-B binding sites, we analyzed whether the presence of sites 8 or 13 could account for some of the difference in CRE activity. To determine how much of the difference in activity was contributed by these sites in vivo, we mutated them and tested GFP-reporter activity of the dimorphic element in transgenic pupae. Dimorphic element activity was reduced to 78 ± 5% of the wild-type CRE by mutation of site 8 (Figure 6B), while site 13 had no affect on activity (Figure 6C). Thus, differences in ABD-B sites account for a portion but not nearly all of the expanded activity of the *D. mel.* dimorphic element.

We ruled out that differences in DSX binding site number contributed to the activity difference between the dimorphic elements because the *D. wil.* element contained both the Dsx1 and Dsx2 sites. We noticed however, that while the two DSX sites were conserved, a two base pair change in the *D. wil.* Dsx1 site caused a reversal of the site's polarity. To determine whether this polarity reversal affected CRE activity, we altered the *D. mel.* Dsx1 site to match that of the *D. wil.* site, and tested this modified element for GFP-reporter activity. This alteration reduced activity to 87 ± 2% of the wild-type element (Figure 6D).

We also tested the effect of site polarity on the activity of the *D. wil.* element by making the reciprocal alteration to the *D. wil.* element, mutating the Dsx1 site to that of the *D. mel.*, and analyzed this modified element for activity in transgenic pupae. Surprisingly, this alteration dramatically increased the activity of the *D. wil.* element from just 1 ± 1% to 34 ± 3% of the wild-type activity of the *D. mel.* element (compare Figures 6G and 6H). Importantly, the increased activity of this modified element was due to the polarity and not the affinity of DSX^F for the site. The DSX protein bound both the *D. mel.* and *D. wil.* sites with equivalent affinity in EMSAs (Figure S4). These results indicated that the polarity of the DSX binding sites also contributed to the divergence of dimorphic CRE activity between *D. wil.* and *D. melanogaster*.

The contribution of both differences in ABD-B binding site number and Dsx1 site polarity to the activity difference between the *D. mel.* and *D. wil.* elements raised the possibility that cumulative mutational changes were responsible for CRE activity divergence. To test this possibility, we combined mutations of ABD-B sites 8 and 13 with mutations reversing the Dsx1 site polarity, and observed that dimorphic element activity was reduced to 66 ± 3% of the wild-type element (Figure 6E), below the level caused by either of the mutations alone. However, additional changes beyond ABD-B binding site number and Dsx1 site polarity must also have contributed to the divergence of CRE activity in A5 and A6.

Other potential modifications to the divergence of dimorphic element activity could involve sites for other transcription factors and/or the spatial arrangement of binding sites. While we do not know yet the identity of any additional transcription factors that directly regulate the dimorphic element, we noted striking differences in the spacing of binding sites between the orthologous elements in the non-conserved regions between the ABD-B and DSX binding sites. The most prominent spacing differences resided between ABD-B site 5 and Dsx1 (58 base pairs), ABD-B sites 8 and 9 (98 base pairs), and Dsx2 and ABD-B site 11 (57 base pairs). We refer to these as regions I, II, and III, respectively

(G and K) When the Dsx2 site was mutated, activity in females was reduced to 34 ± 3% (G), while male activity was unchanged (K).
(H and L) When both the Dsx1 and Dsx2 sites were mutated, reporter activity in females was reduced to 24 ± 1% (H), and increased to 53 ± 3% in males (L).

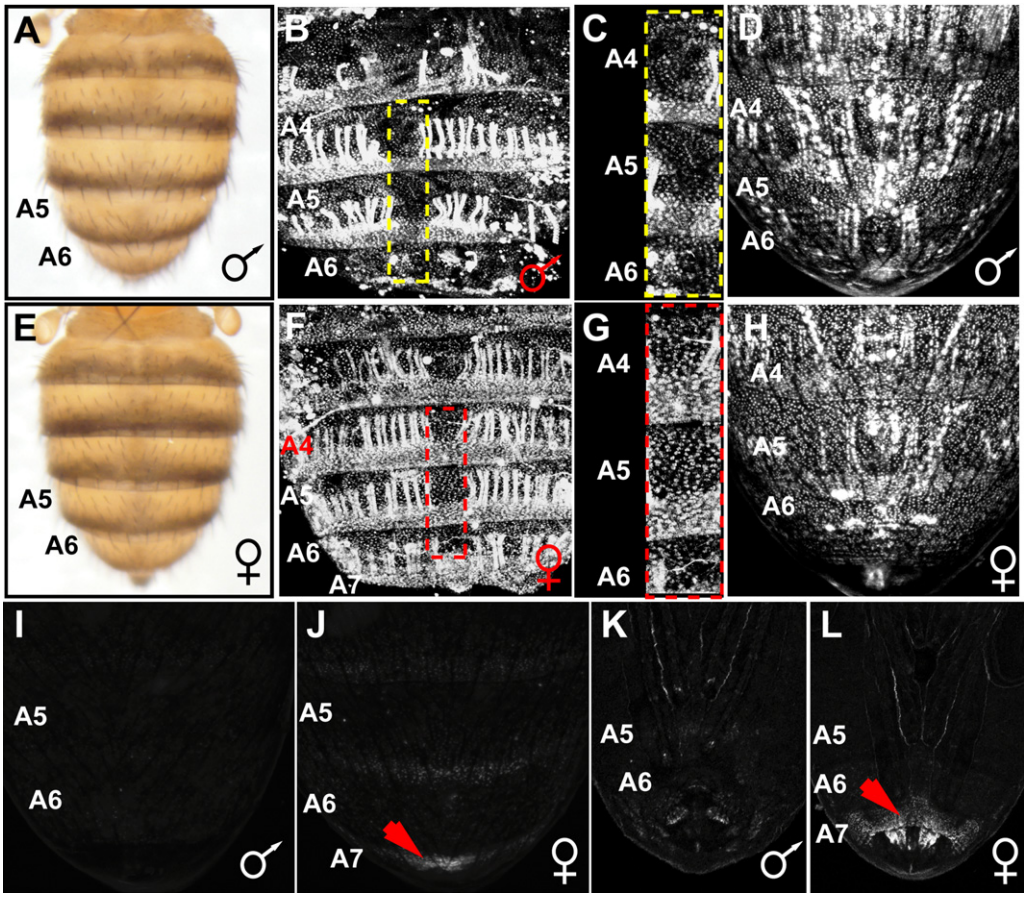

Figure 5. The Dimorphic Element Has a Deep Ancestry

(A) Dorsal views of *D. wil.* male abdomen. Pigmentation of abdominal tergites on segments A2–A6 is limited to a posterior stripe.

(B, C, F, and G) Bab1 expression in pupal abdomens at 65 hr APF (a developmental time point equivalent to 72 hr APF assayed for *D. mel.*).

(B) Bab1 is expressed in segments A2–A6 (A2 not shown) of male pupae. Expression was also observed in longitudinal abdominal muscles.

(C) High-magnification view of (yellow box [B]) dorsal midline showing equivalent levels of Bab1 in segments A4–A6.

(D) The *D. wil.* anterior element drove GFP-reporter expression in segments A2–A6 of males (A2 not shown).

(E) Dorsal view of *D. wil.* female abdomen. The pigmentation pattern is identical to that of the male (compare with [A]).

(F) Bab1 is expressed in segments A2–A7 of female pupae.

(G) High-magnification view of (red box [F]) dorsal midline showing equivalent levels of Bab1 in segments A4–A6.

(H) The *D. wil.* anterior element drove reporter expression in segments A2–A7 of females (A2 and A3 not shown).

(I) The *D. wil.* dimorphic element does not activate reporter expression in posterior segments of males (dorsal view).

(J) The *D. wil.* dimorphic element drove expression in the A7 segment of females (red arrow; dorsal view).

(K) Ventral view showing the absence of *D. wil.* dimorphic element reporter activity in posterior segments of males.

(L) Ventral view showing reporter expression driven by the *D. wil.* dimorphic element in female segment A7 (red arrow), but not in the anterior A6 segment.

(Figure 7B), where in each region the *D. wil.* element possesses the greater spacing between binding sites. Compared to members of outgroups, the reduced spacing in regions I–III in *D. mel* is a general feature shared among the *melanogaster* species group (Figure S5). To test whether differences in the spatial topology of binding sites affected dimorphic element activity, we inserted the additional *D. wil.* sequences residing in regions I–III into the orthologous positions of the *D. mel.* element (Figures 6I–6L), making the distances between the adjacent binding sites in the *D. mel* element equal to those of the *D. wil.* dimorphic element. We found that the activity of the *D. mel.* dimorphic CRE was reduced to 62 ± 3%, 41 ± 3%, and 44 ± 4% of the wild-type, respectively when the *D. wil.* sequences were inserted into regions I, II, and I–III (Figures 6A, 6I, 6J, and 6L). These effects appeared to be due to changes in spacing and not the insertion of sequences that were bound by repressors, because the reciprocal deletion of these sequences in the *D. wil.* element led to no net gain in CRE activity (data not shown). Interestingly, insertion of the *D. wil.* sequence into region III increased the activity of the wild-type dimorphic element by 37 ± 3% (Figure 6K). This result demonstrated that the regulatory activity of the *D. mel.* element is not at a maximum and is further evidence of how changes in binding site topology can increase or decrease CRE activity.

Together, these results reveal that the expansion of the activity of the *D. mel.* dimorphic element into anterior abdominal segments did not result from the gain of ABD-B or DSX binding

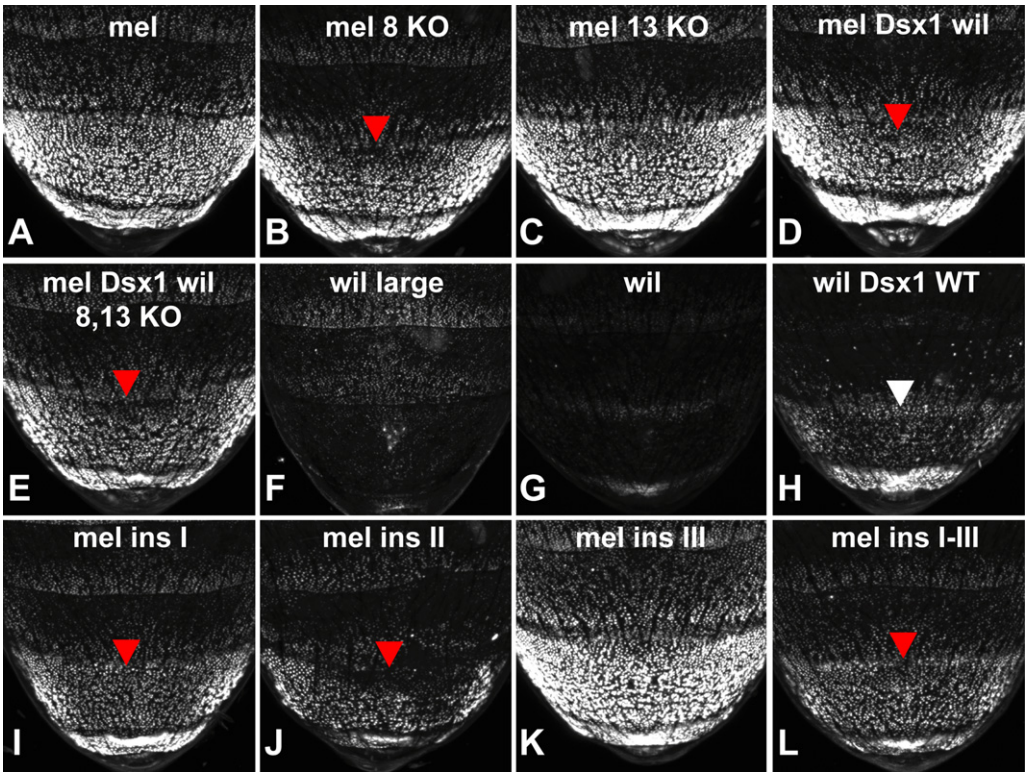

Figure 6. *bab* Expression Evolved via Remodeling of the Dimorphic Element

In all panels, GFP-reporter expression mediated by *D. mel.* (A–E and I–L) and *D. wil.* (F–H) dimorphic elements was assayed in transgenic female pupae at 75 hr APF. Activity measurements are represented as the % of the wild-type (mel) female A6 mean ± SEM. Red arrow heads point to dorsal midline regions of A6 where reporter activity was reduced by modification of the *D. mel.* element. White arrow head points to area of segment A6 where reporter activity was increased by modification of the *D. wil.* element.

(A) Reporter expression driven by the wild-type *D. mel.* dimorphic element.

(B) Mutation of ABD-B site 8 reduced reporter activity to 78 ± 5%.

(C) Mutation of ABD-B site 13 had no measurable affect on reporter activity.

(D) Reversal of the Dsx1 site polarity in the *D. mel.* element reduced reporter activity to 87 ± 2%.

(E) Reversal of Dsx1 site polarity combined with mutation of ABD-B sites 8 and 13 reduced reporter activity to 66 ± 3%.

(F) Reporter expression driven by the *D. wil.* dimorphic element that includes site 14.

(G) Reporter expression driven by the wild-type *D. wil.* dimorphic element.

(H) Reversal of Dsx1 site polarity in the *D. wil.* element resulted in a dramatic gain of reporter activity in segment A6. Activity increased from 1 ± 1% to 34 ± 3% of the wild-type *D. mel* element.

(I) Insertion of 58 base pairs between ABD-B site 5 and Dsx1 site (region I) reduced reporter expression to 62 ± 3%.

(J) Insertion of 98 base pairs between ABD-B sites 8 and 9 (region II) reduced reporter expression to 41 ± 3%.

(K) Insertion of 57 base pairs between the Dsx2 site and ABD-B site 11 (region III) increased the activity of the wild-type dimorphic element by 37 ± 3%.

(L) Insertion of 58, 98, and 57 base pairs into regions I-III respectively, reduced dimorphic element activity to 44 ± 4%.

sites. Rather this expanded activity resulted from an amalgam of changes in the CRE involving the number, polarity, and topology (spacing) of binding sites, what we describe as the molecular "remodeling" of a pre-existing DSX- and ABD-B-regulated CRE.

DISCUSSION

We have shown that *bab* expression in the abdominal epidermis is regulated by two separate CREs, one of which directs gene expression in the anterior abdomen of both sexes, and a second, dimorphic element that regulates female-specific gene expression in segments A5–A7. The dimorphic element, when bound by ABD-B and sex-specific isoforms of the DSX protein, acts as a genetic switch that allows pigmentation in males and represses pigmentation in females. We found that changes in the activities of both CREs have evolved in the course of the origin of the trait from a monomorphic ancestor. Furthermore, we demonstrated that dimorphic CRE function evolved by multiple fine-scale changes within the CRE. These results bear on our understanding of how sexually dimorphic traits develop, how new sex- and segment-restricted traits arise, and how CRE functions evolve.

A Genetic Switch Controlling Sexually Dimorphic Traits

Sex-restricted traits are the product of differences in gene expression between sexes, therefore, understanding how such

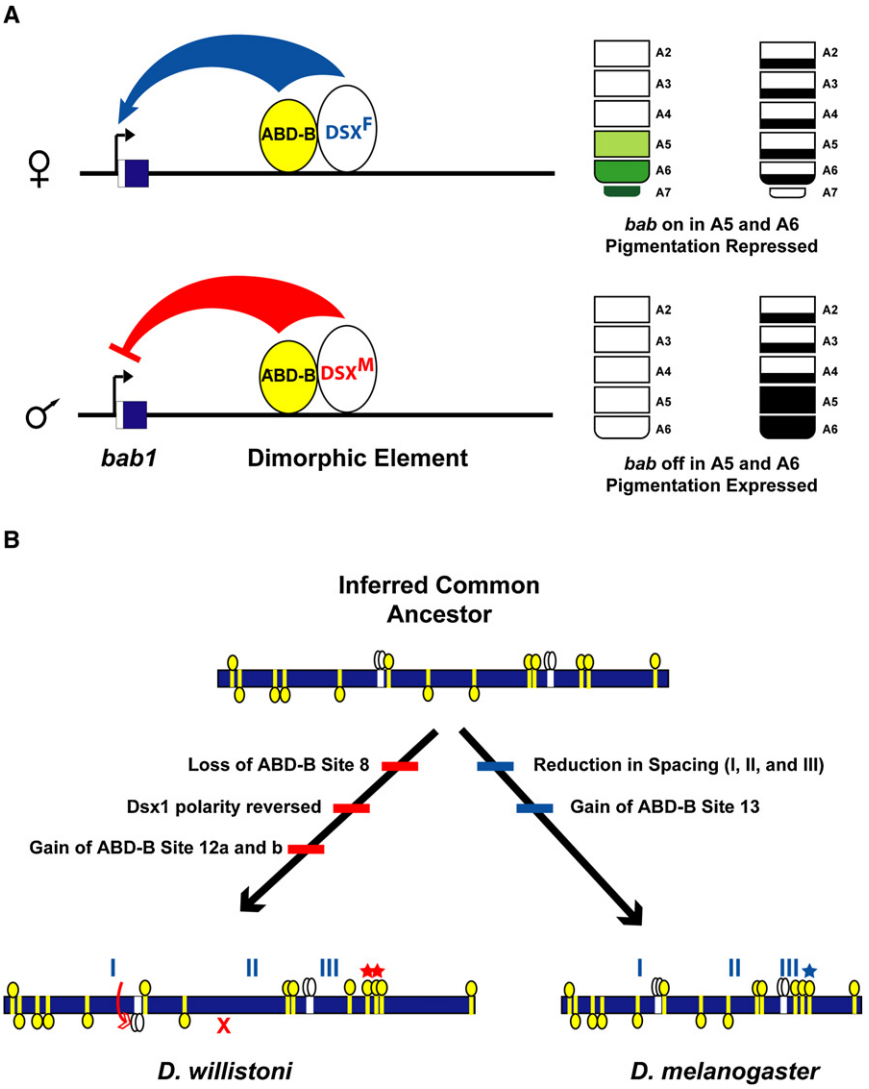

Figure 7. Model for the Operation and Evolution of the Dimorphic Genetic Switch

(A) The operation of the switch. Expression of *bab* in the posterior abdominal segments A5–A7 of females is mediated by the combined inputs of the segment-specific HOX protein ABD-B and the female-specific isoform DSX^F. Expression of *bab* results in the repression of full tergite pigmentation in these segments. Expression of *bab* in male segments A5 and A6 is repressed by the male-specific isoform DSX^M. The absence of *bab* expression in these segments allows for the development of fully-pigmented tergites.

(B) The evolution of the switch. Schematic depiction of the evolution of the dimorphic element from the inferred common ancestor of *D. melanogaster* and *D. willistoni*. Yellow boxes indicate binding sites for ABD-B and white boxes indicate DSX binding sites. Yellow and white ovals represent ABD-B and DSX protein monomers respectively. The common ancestral CRE contained two and thirteen orthologous binding sites for DSX and ABD-B, respectively. In the lineage leading to *D. wil.*, ABD-B site 8 was lost, the polarity of Dsx1 was reversed (red arrow) and candidate ABD-B binding sites 12a and 12b were gained (red stars). In the lineage leading to *D. mel.*, interbinding site spacing was reduced in regions I, II, and III, and ABD-B site 13 was gained (blue star), which collectively contributed to the higher level of gene expression in female segments A5 and A6.

traits develop requires the identification of those genes with sex-limited expression and elucidation of the genetic and molecular mechanisms governing their regulation. We showed that dimorphic *bab* expression is regulated by a discrete CRE whose activity is combinatorally regulated by the direct inputs of both region- (ABD-B) and sex-specific (DSX) transcription factors. In females, ABD-B acts in concert with the DSX^F isoform through binding sites in the dimorphic element to activate *bab* expression in the posterior segments. Whereas in males, ABD-B activity is overridden by the repressive activity of the DSX^M isoform which binds to the same sites as DSX^F and hence, permits the formation of the male-specific posterior pigmentation (Figure 7A).

The genetic pathways that regulate sex-determination and sexual differentiation differ greatly across the animal kingdom, so this mode of male-specific trait regulation in *Drosophila* may not apply in detail to other animals. However, the integration of region- and sex-specific regulatory inputs must be a requirement for the production of dimorphic traits. We suggest that the integration of such combinatorial inputs by cis-regulatory elements, as we have demonstrated for *bab*, is a general feature of genetic switches within the pathways regulating the production of dimorphic traits.

The Evolution of a New Dimorphic Trait

The origins of sexually dimorphic traits have long been of central interest in evolutionary biology. One of the key questions that Darwin (Darwin, 1871) grappled with, as have many others subsequently (Fisher, 1930), was whether dimorphic traits are limited to one sex at their origin, or whether these traits first appear in both sexes and then become restricted to one sex. This question has been particularly important and challenging in terms of genetics and evolutionary theory, as it has not been resolved previously how the effects of mutations could be restricted to one sex.

In the simplest genetic scenarios of sexual dimorphism, male-limited traits are the products of the male-limited expression of specific genes. The main evolutionary question then, as it has been phrased in classical genetic terms, is whether male-limited gene expression evolves via: (1) ‘‘alleles’’ that are expressed only in males; or (2) alleles expressed in both sexes which are then suppressed in females or promoted in males (Coyne et al., 2008). The elucidation of the regulation and evolution of male-specific pigmentation provides a unique opportunity to reconstruct the genetic path of the evolution of a dimorphic trait.

Although posterior male-specific pigmentation is a relatively simple, two-dimensional morphological trait, it is clear that it did not originate via just one of the alternative genetic paths above. Rather, the evolution of this trait has involved three paths - the evolution of male-limited gene expression, of female-limited gene expression, and of non-sex-restricted gene expression. Specifically, we have shown here that in the course of the evolution from a monomorphically pigmented ancestor, the activity of the female-specific *bab* dimorphic CRE expanded into segments A6 and A5 and that the activity of the monomorphic *bab* anterior CRE retreated from segments A6 and A5 of both sexes. These two combined changes produced the sex-specific repression of *bab* expression in male segments A5 and A6. In addition, in previous work we showed that the *yellow* pigmentation gene gained high-level expression in segments A5 and A6 via the acquisition of ABD-B binding sites in a specific *yellow* gene CRE (Jeong et al., 2006), whose activity was male-limited due to repression by Bab (which is apparently indirect).

It is important to underscore that none of the genes in this newly-evolved regulatory circuit are globally restricted in their expression to one sex. Rather, the sex-specific features of their expression are controlled by modular CREs that are physically separate from those controlling gene expression in other developing body regions. The properties of these CREs resolve the question of how the effects of mutations can be restricted to one sex. Namely, mutations in a CRE that is under the direct (the female-specific *bab* dimorphic element) or indirect (the male-specific *yellow* CRE*)* control of an effector of sex determination will have sex-limited effects on gene expression. The findings here are a further demonstration of the general principle of how the modular CREs of pleiotropic genes enable the modification of gene expression in and morphology of one body part independent of other body parts, or in this case, the same body part in the opposite sex (Carroll, 2005, 2008).

It is also notable that none of the CREs we have analyzed are new to the dimorphically pigmented *melanogaster* species group. It is clear, then, that the ancestral dimorphic CRE was active in segment A7 and modified to govern sexually dimorphic pigmentation in segments A6 and A5. Thus, in this example, we see that one path to evolving a new dimorphic trait is via the co-option of genetic components that regulate other pre-existing dimorphic traits.

Remodeling of CREs and the Target Size of Functionally Relevant Mutations

One of the major questions concerning the evolution of gene expression is how new gene expression patterns arise. The two most obvious mechanisms would appear to be the gain of new regulatory elements or the gain of new transcription factor-CRE linkages. While the deep ancestry of the dimorphic element ruled out the former, we expected that the novel sex- and segment-specific regulation of this CRE by DSX and ABD-B in the *D. mel.* lineage would require the gain of binding sites for these two transcription factors. However, we found that the both DSX binding sites and most ABD-B sites were present in *D. wil.* and other monomorphic species and therefore were present in the last common ancestor of both monomorphic and dimorphic species. Thus, the expansion of the dimorphic CRE activity was not due to the wholesale gain of new DSX and ABD-B binding sites.

Rather, we discovered that the expanded, high level activity of the *D. mel.* dimorphic CRE in segments A6 and A5, relative to the A7-restricted activity of the *D. wil*. element, was due to an amalgam of changes involving the number, polarity, and topology of transcription factor binding sites. The evolution of dimorphic CRE activity demonstrates how changes beyond the simple gain or loss of binding sites shape CRE evolution. Similarly, we recently showed that changes in the topology and helical phasing of transcription factor binding sites shaped the evolution of a genetic switch controlling galactose utilization in yeast (Hittinger and Carroll, 2007). Our studies strongly support the view that the relationship between function and sequence variation in CREs is complex (Balhoff and Wray, 2005). A vast body of work on eukaryotic and prokaryotic transcriptional regulation has shown that binding site polarity and spacing influences the output of regulatory elements. Therefore, we suggest that one important, but generally unappreciated, class of functionally relevant mutations in CRE and trait evolution involves sequences outside of transcription factor binding sites. CREs thus present a very large target area for potential functionally relevant mutations that quantitatively modulate gene expression and trait development.

Hox Genes and the Evolution of Axial Traits

Finally, we suggest that our observations concerning the mechanisms underlying the expansion of dimorphic CRE activity help to shed light on another general aspect of the evolution of animal body plans - the evolution of segmental traits. A large number of studies have demonstrated that some of the major differences among arthropod and vertebrate body plans have involved evolutionary shifts in the spatial boundaries of gene expression along the main body axis (Carroll, 2005). However, the path by which such gene expression patterns are shifted has not been elucidated in any molecular detail. We submit that the expansion of the activity of the dimorphic element from the A7 segment into A6 and A5 is a model of this process. The remodeling of the dimorphic CRE in the course of evolution illustrates that one way such shifts can be accomplished is through numerous small, quantitative incremental changes in the activity of *Hox*-regulated CREs.

EXPERIMENTAL PROCEDURES

Fly Stocks

The *Canton*^{S} strain of *D. melanogaster* (wild-type) and *D. willistoni* stocks were obtained from the Tucson Stock Center. Genetic analyses were performed using the following alleles: *Abd-B*^{Mcp} (Duncan, 1987) and *Abd-B*$^{lab9\text{-}Tab}$ (Celniker and Lewis, 1987), two gain-of-function alleles that drive ectopic *Abd-B* expression in segment A4 and A3-A4 respectively; *dsx*$^{D+R3}$, a *dsx* null allele, and; *dsx*^{D}, a mutant in which *dsx* RNA is altered from production of the female *dsx*^{F} transcript to the production of male *dsx*^{M} transcript, resulting in female intersexes (Duncan and Kaufman, 1975).

Reporter Constructs and Transgenic Fly Production

All DNA sequences used in GFP-reporter constructs were cloned into either p-element or site-specific transformation vectors. Additional information on the production of constructs, transgenic lines, and scoring of reporter expression phenotypes are provided in the Supplemental Experimental Procedures and Tables S1–S3.

DNA-Binding Analyses

DNaseI footprinting reactions and EMSAs were performed as previously described (Jeong et al., 2006) using a GST-DSX DNA Binding Domain (DBD) fusion protein (Supplemental Experimental Procedures). PAGE-purified oligos used in EMSAs are listed in Table S4.

Immunohistochemistry

Immunohistochemistry of pupal abdominal epidermis was performed as previously described (Gompel and Carroll, 2003) using an affinity purified anti-Bab1 antibody (Supplementary Experimental Procedures) on *D. mel.* and *D. wil.* specimens, that were dissected at 72 and 65 hr after puparium formation (APF) respectively. Detailed protocol is available at: http://www.molbio.wisc.edu/carroll/methods//methods.html.

Measurement of Relative Fluorescence Intensity

The relative fluorescence intensities for A6 reporter expression was determined as previously described (Jeong et al., 2008) with modifications described in the Supplemental Experimental Procedures.

Imaging of Fly Abdomens

Images of adult abdomens were taken using an Olympus SZX16 Zoom Stereo Microscope equipped with an Olympus DP71 microscope digital camera. Pupal immunohistochemistry and transgenic GFP-reporter line samples were imaged using an Olympus Fluoview FV 1000 confocal microscope and software. Pupae from transgenic reporter lines were dissected from pupal cases at 72-78 hr APF and mounted in Halocarbon 700 oil for confocal analysis.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, five figures, and four tables and can be found with this article online at http://www.cell.com/cgi/content/full/134/4/610/DC1/.

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