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Evolution of *yellow* Gene Regulation and Pigmentation in *Drosophila*

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Summary

Background: Changes in developmental gene expression are central to phenotypic evolution, but the genetic mechanisms underlying these changes are not well understood. Interspecific differences in gene expression can arise from evolutionary changes in *cis*-regulatory DNA and/or in the expression of *trans*-acting regulatory proteins, but few case studies have distinguished between these mechanisms. Here, we compare the regulation of the *yellow* gene, which is required for melanization, among distantly related *Drosophila* species with different pigment patterns and determine the phenotypic effects of divergent Yellow expression.

Results: Yellow expression has diverged among *D. melanogaster*, *D. subobscura*, and *D. virilis* and, in all cases, correlates with the distribution of black melanin. Species-specific Yellow expression patterns were retained in *D. melanogaster* transformants carrying the *D. subobscura* and *D. virilis yellow* genes, indicating that sequence evolution within the *yellow* gene underlies the divergence of Yellow expression. Evolutionary changes in the activity of orthologous *cis*-regulatory elements are responsible for differences in abdominal Yellow expression; however, *cis*-regulatory element evolution is not the sole cause of divergent Yellow expression patterns. Transformation of the *D. melanogaster yellow* gene into *D. virilis* altered its expression pattern, indicating that *trans*-acting factors that regulate the *D. melanogaster yellow* gene have also diverged between these two species. Finally, we found that the phenotypic effects of evolutionary changes in Yellow expression depend on epistatic interactions with other genes.

Conclusions: Evolutionary changes in Yellow expression correlate with divergent melanin patterns and are a result of evolution in both *cis*- and *trans*-regulation. These changes were likely necessary for the divergence of pigmentation, but evolutionary changes in other genes were also required.

Introduction

A major challenge of biology is understanding the genetic basis of evolutionary change. Genetic analyses of model organisms have identified a number of "candidate" genes that may have contributed to the evolution of phenotypic differences between species. Mutations in these genes produce phenotypes that resemble those of other species, and this has prompted hypotheses that similar genetic changes may have given rise to existing interspecific differences (for examples in *Drosophila*, see [1–5]). To test these hypotheses, the regulation and function of some of these candidate genes have been compared among species [2, 5]. Differences in the expression of developmentally important genes have been identified that correlate with shifts in morphology, strongly suggesting that the evolution of gene regulation was a major contributor to phenotypic divergence (reviewed in [6–8]). The specific genetic changes responsible for particular differences in gene expression remain largely unknown.

Gene transcription is controlled by the physical interaction of transcription factor proteins with *cis*-regulatory DNA, where the sequence of the *cis*-regulatory DNA determines which transcription factors regulate the gene (reviewed in [7]). Thus, gene expression can be altered by changing either the spatial distribution or concentration of *trans*-acting transcription factors or the sequence of *cis*-regulatory DNA. Comparing the expression of divergent, orthologous genes in a common genetic background can distinguish between these changes, and such comparisons have been made in a handful of studies using either interspecific genetics [2, 5, 9, 10] or transgenic technologies [11–18].

For example, evolutionary changes within the *Drosophila Ultrabithorax* (*Ubx*) and *ovo* genes have been examined in interspecific hybrids [2, 5]. Divergent expression patterns of *Ubx* and *ovo* correlate with changes in specific patterns of leg trichomes and larval hairs, respectively, that they regulate. In hybrid genetic backgrounds lacking endogenous *Ubx* or *ovo* gene function, species-specific alleles of these genes produce phenotypes most similar to the species from which the allele was derived, suggesting that changes within these genes (i.e., in *cis*-regulatory elements controlling gene expression) are responsible for the species-specific patterns of gene expression and morphology.

Comparisons of gene expression in transgenic animals carrying orthologous genes have also demonstrated evolutionary changes in *cis*-regulatory elements (reviewed in [11]). Species-specific expression patterns of the *Drosophila alcohol dehydrogenase* (*Adh*) and *glucose dehydrogenase* (*Gld*) genes were retained in *D. melanogaster* transformants [12–17], indicating that the function of *cis*-regulatory sequences had diverged. Similarly, a comparison of the activity of orthologous *Hoxc8* *cis*-regulatory regions from the mouse and chicken in transgenic mice indicated that divergence of these *cis*-regulatory sequences was responsible for interspecific differences in gene expression [18].

Rapidly evolving melanin patterns in the genus *Drosophila* offer an excellent opportunity to investigate the genetic and molecular bases of phenotypic evolution. Melanin patterns play a major role in important physiological and ecological processes such as thermoregulation, mimicry, camouflage, and mate choice (reviewed

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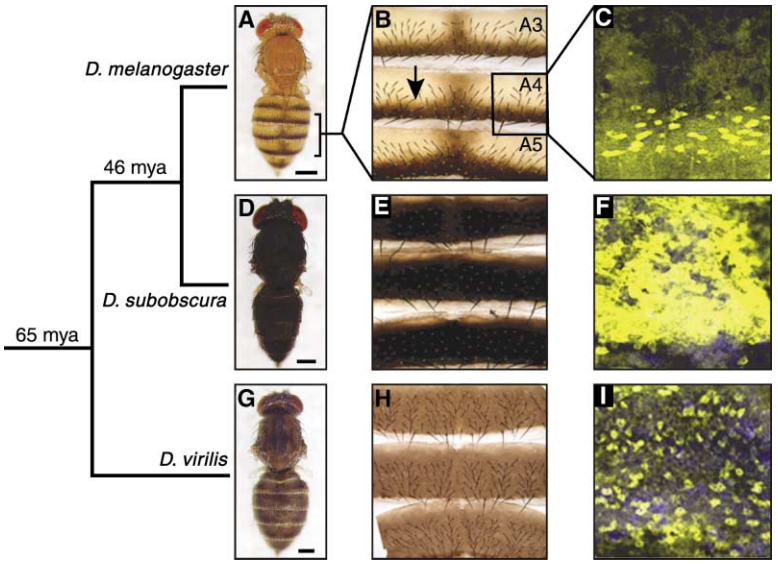

Figure 1. Evolution of Yellow Expression Correlates with the Divergence of Body Pigmentation among *Drosophila* Species

(A–I) (A and B) *D. melanogaster* produces a dark stripe of melanin near the posterior edge of each abdominal tergite (arrow). (C) Yellow protein is present predominantly in the cells that will produce this stripe. (D, E, G, and H) In *D. subobscura* and *D. virilis*, the abdomen is more uniformly pigmented, with more melanin produced in *D. subobscura*. (F and I) Yellow protein is also present throughout the abdominal tergites of both species, with higher levels of protein expressed in (F) *D. subobscura*. *D. melanogaster* males have additional melanization (and Yellow expression) in the A5 and A6 tergites, whereas *D. subobscura* and *D. virilis* do not display sexually dimorphic pigmentation or Yellow expression (data not shown). Phylogenetic relationships and estimated divergence times in millions of years ago (mya) [63] are shown on the left. (A, D, and G) Wings were removed to better show body pigmentation, and the relative size of adult files is indicated by a scale bar in the lower right corner. (B, E, and H) Abdominal tergites from segments A3–A5, with anterior at the top and the dorsal midline in the center. The same abdominal segments are shown in Figures 2, 4, and 5. (C, F, and I) Yellow expression in the lateral A4 tergite at approximately 72 hr APF. The nonuniform distribution of the Yellow protein in abdominal epidermal cells is due to posttranscriptional regulation of the *yellow* gene [28].

in [19]), suggesting that differences in pigmentation may have been shaped primarily by natural selection. In the *Drosophila* lineage, many different melanin patterns have evolved (e.g., [20–24]), and changes in the regulation of melanin patterning genes may underlie pigmentation divergence. Recently, some of the genetic and molecular mechanisms controlling the spatial distribution of melanin in the abdomen of *D. melanogaster* have been elucidated [24–31], which has made it possible to compare the regulation of melanin patterning among *Drosophila* species at a molecular level.

Here, we investigate the role of one candidate gene, *yellow*, in the evolution of *Drosophila* pigmentation. In *D. melanogaster*, the *yellow* gene is required for the formation of black melanin [32], Yellow protein expression correlates with melanin patterns [28, 33, 34], and changes in Yellow expression can significantly alter pigmentation [28]. *yellow* mutants isolated in other *Drosophila* species are also unable to produce melanin, indicating that the function of the *yellow* gene is conserved among Drosophilids [35]. Changes in Yellow expression may therefore have been required for the divergence of melanin patterns and could have arisen through sequence evolution of either the *yellow* gene or of genes that encode *trans*-regulatory proteins controlling Yellow expression. If Yellow expression differences were sufficient to alter pigmentation, then selection for novel melanin patterns may have favored novel Yellow expression patterns.

In this work, we compare the regulation of the *yellow* genes from *D. melanogaster*, *D. subobscura*, and *D. virilis* and find that Yellow expression has diverged among these species; this finding correlates with the distribution of melanin in all cases. Using transgenic flies carrying heterologous *yellow* genes, we show that interspecific differences in Yellow expression are due to evolutionary changes in both *cis*-regulatory sequences and *trans*-regulatory proteins, with the *cis*-regulatory changes localized to a discrete 5′ regulatory region. Changes in the expression pattern of Yellow only alter pigmentation in some genetic backgrounds, indicating that evolutionary changes at other loci have also contributed to the divergence of melanin patterns.

Results

D. melanogaster, *D. subobscura*, and *D. virilis* are distantly related species with dramatic differences in the spatial distribution and intensity of abdominal melanization (Figure 1). In *D. melanogaster*, each abdominal tergite displays a black melanin stripe near the posterior edge of each segment (Figures 1A and 1B, arrow). In contrast, both *D. subobscura* and *D. virilis* produce melanin throughout each abdominal tergite, with darker pigmentation in *D. subobscura* than *D. virilis* (compare Figures 1D and 1E with Figures 1G and 1H). Here, we investigate whether evolutionary changes in *yellow*, a melanin patterning gene, contribute to these interspecific differences in pigmentation.

Yellow Expression Correlates with Divergent Pigment Patterns

Expression of the Yellow protein correlates with melanization in *D. melanogaster* [28, 33, 34] (Figures 1A–1C). To determine if a similar correlation exists in other *Drosophila* species, we examined the distribution of the Yellow protein in developing abdomens of *D. subobscura* and *D. virilis*. In both species, Yellow protein was present throughout the abdominal tergite, with higher levels of Yellow expression in *D. subobscura* than in *D. virilis* (Figures 1F and 1I). This expression matches the pattern and intensity of black melanin in the adult flies (Figures 1E and 1H). Because the *yellow* gene is required for the production of melanin in all three species [32,

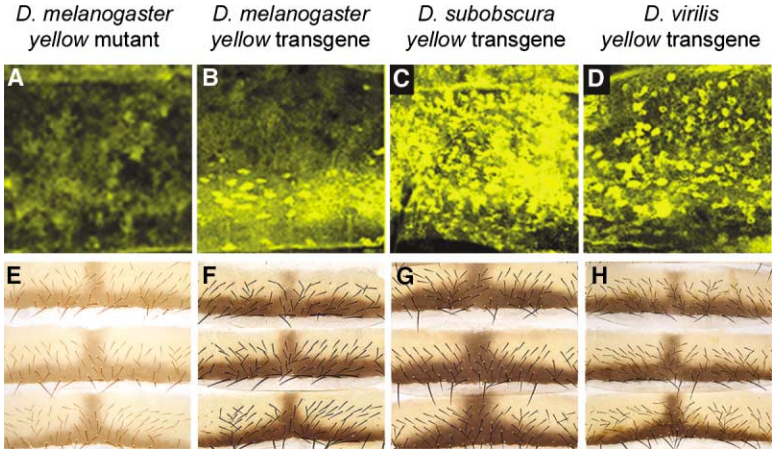

Figure 2. Evolutionary Changes within the *yellow* Gene Are Responsible for Interspecific Differences in Yellow Expression

(A) The *D. melanogaster yellow* mutant strain used as the transformation host does not produce Yellow protein. (The faint yellow color is tissue autofluorescence).

(B–D) *D. melanogaster* transformants carrying the (B) *D. melanogaster*, (C) *D. subobscura*, and (D) *D. virilis yellow* genes express Yellow protein in patterns that are indistinguishable from the native Yellow expression pattern in the species from which the *yellow* gene was derived (compare with Figures 1C, 1F, and 1I).

(E–H) Heterologous *yellow* transgenes are able to rescue the melanin production defect of *D. melanogaster yellow* mutants; however, the significant differences in the pattern of Yellow expression (B–D), do not cause dramatic differences in adult melanin patterns (F–H). (G) The broadening of the midline pigment stripe caused by the *D. subobscura yellow* transgene is also caused by ectopic expression of high levels of *D. melanogaster* Yellow in these cells [28]. (E) Yellow is only required for black pigmentation, and the brown and tan pigments present in *yellow* mutants are produced by alternate branches of the melanin synthesis pathway [28]. Yellow expression is shown at the same developmental stage and in the same segment as in Figure 1.

36, 37], and its expression has evolved in concert with melanin patterns, evolutionary changes in Yellow expression may have played a critical role in pigmentation divergence.

cis-Regulatory Evolution Contributes to Interspecific Differences in Yellow Expression

Divergence of gene expression patterns can be due to evolutionary changes in *cis*-regulatory sequences and/or *trans*-regulatory proteins. To determine which of these changes is responsible for the divergence of Yellow expression, we compared the expression of the *D. melanogaster*, *D. subobscura*, and *D. virilis yellow* genes in a common environment of transcription factors by transforming the *yellow* gene from each species into *D. melanogaster*. The *D. melanogaster* and *D. subobscura yellow* genes had been previously cloned [38, 39], and the *D. virilis yellow* gene was isolated for this study (see the Experimental Procedures). The *yellow* mutant strain of *D. melanogaster* used as the transformation host did not produce Yellow protein (Figure 2A), ensuring that any Yellow protein detected in the transformant was derived from the *yellow* transgene.

In all cases, the *yellow* transgenes retained their species-specific expression patterns when transformed into *D. melanogaster* (Figures 2B–2D, also see Figures 1C, 1F, and 1I). In the abdomen, the *D. melanogaster* transgene was expressed in the cells that produce the pigment stripe (Figure 2B), whereas the *D. subobscura* and *D. virilis* transgenes were expressed throughout the abdominal tergite, with higher levels of expression from the *D. subobscura* transgene (Figures 2C and 2D). The proper regulation of heterologous *yellow* genes in *D. melanogaster* indicates that the expression of at least some of the transcription factors necessary for their expression are likely to be conserved in *D. melanogaster*, and that expression differences are due to evolutionary changes in sequences included in the transgene.

Each transgene contained the entire *yellow* gene from each species; thus, the observed differences in Yellow protein production could be a result of differences in transcription controlled by *cis*-regulatory sequences or in posttranscriptional processing (i.e., mRNA stability, translation efficiency, protein stability) controlled by the untranslated regions and coding sequence. To test if evolutionary changes in *yellow* transcription were responsible for differences in Yellow protein expression, we better characterized the *cis*-regulatory elements of the *D. melanogaster yellow* gene and determined the function of orthologous sequences from *D. subobscura*.

Abdominal Expression of the *D. melanogaster yellow* Gene Is Controlled by a Discrete *cis*-Regulatory Element

Previous work in *D. melanogaster* identified regions of the *yellow* gene that are required for Yellow expression in specific tissues such as the wing, body (head, abdomen, and thorax), and bristles [38, 40, 41]. We sought to better understand the functions of these *cis*-regulatory regions by testing if they were sufficient to control gene transcription in these tissues.

Putative wing and body enhancer sequences (Figure 3A) were inserted upstream of a weak promoter controlling the expression of the easily visualized green fluorescent protein (GFP) and were transformed into *D. melanogaster*. The 800 bp required for *yellow* function in the wing were sufficient to direct GFP expression in this tissue (Figure 3C). Additional GFP expression was observed in a cell associated with each bristle and at low levels in abdominal epidermal cells (Figure 3C). The 1.4 kb of sequence required for *yellow* function in the body was sufficient to activate transcription exclusively in the epidermal cells of the head, thorax, and abdomen (Figure 3D). The pattern of reporter gene expression in the abdomen, however, was not identical to the distribution of the native Yellow protein in these cells.

In the abdomen of *D. melanogaster*, Yellow protein expression is temporally dynamic, with the protein initially present in cells throughout the abdominal tergite and later restricted to cells that produce the adult pig-

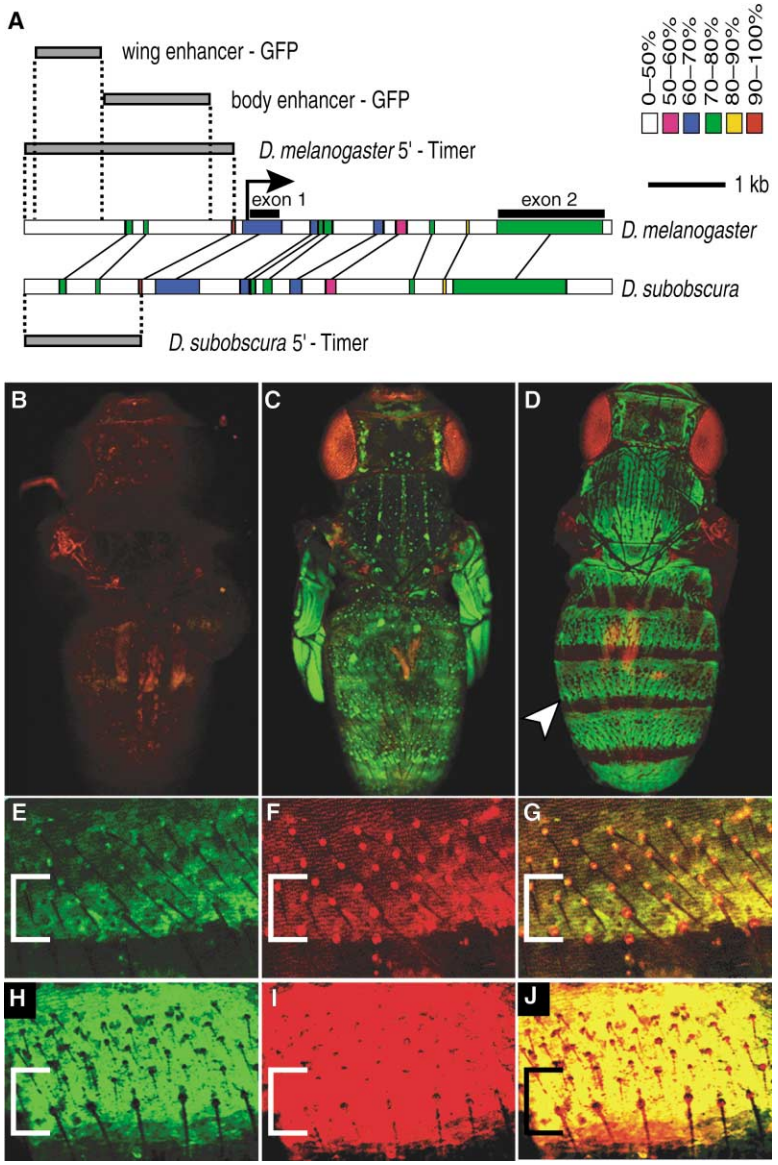

Figure 3. The Function of a Discrete *cis*-Regulatory Region of *yellow* Has Diverged between *D. melanogaster* and *D. subobscura*

(A) A schematic indicating the percentage of identical nucleotides in conserved sequence blocks between the aligned *D. melanogaster* and *D. subobscura yellow* genes. The locations of exons (black bars) and the fragments used to make the GFP and Timer reporter genes are also shown (gray bars). An arrow indicates the transcriptional start site.

(B) Autofluorescence of the *D. melanogaster yellow, white* transformation host.

(C) The wing-GFP reporter gene expresses GFP (shown in green) in the wings, in bristle cells, and at a low level throughout the abdominal epidermis.

(D) The body-GFP reporter gene produces GFP in the epidermal cells of the head, thorax, and abdomen, with elevated levels near the posterior edge of each abdominal tergite (arrowhead). Red eyes in (C) and (D) are due to autofluorescence of eye pigments.

(E–G) Expression of the *D. melanogaster* 5′-Timer reporter gene in the abdominal tergite of a pharate adult is transcriptionally refined to a posterior stripe.

(H–J) The *D. subobscura* 5′-Timer reporter gene does not show this refinement and produces significantly higher levels of Timer protein than the *D. melanogaster* reporter gene. Confocal laser intensity was reduced 5-fold in (H)–(J) relative to (E)–(G). Younger Timer protein is shown in green (E, G, H, and J), and older Timer protein is shown in red (F, G, I, and J). Autofluorescence of cuticular structures and bristle socket cells is also red. (E–J) Flies were incubated at 37°C for 6 hr prior to dissection; half of the A4 tergite is shown, with the lateral edge at the left and the dorsal midline at the right. The bracket indicates cells that will produce the pigment stripe in *D. melanogaster*.

ment stripe [28] (Figure 1C shows the restricted pattern of Yellow expression). The body enhancer reporter gene showed GFP expression throughout the abdominal tergite during all stages examined (Figure 3D), with slightly elevated levels of GFP expression in cells underlying the adult pigment stripe immediately prior to eclosion (arrow). The refinement of Yellow expression in the abdomen is due to temporal changes in the transcription of *yellow* [28], suggesting that the reporter gene either lacks the sequences necessary for that refinement, or that the degradation of GFP is slower than the degradation of Yellow, effectively masking rapid temporal changes in transcription.

To take both possibilities into account, another reporter gene was constructed that included sequences surrounding the original body enhancer (2.7 kb total, including the entire wing enhancer, Figure 3A) and used the recently developed "Timer" protein to report transcriptional activity. Timer is a mutant of the DsRed protein reported to fluoresce green for 3 hr after synthesis, and then red for up to 24 hr [42]. This is the first use of the Timer protein in *Drosophila*, and although the time to maturation of both green and red fluorescence is longer than in other systems (see the Experimental Procedures), the younger green Timer isoform still has a shorter half-life than GFP, making it more sensitive to temporal changes in transcription. In pharate adult flies, the younger green Timer isoform was present predominantly in abdominal epidermal cells that will produce the pigment band (Figures 3E–3G), similar to the final expression pattern of the native *D. melanogaster* Yellow protein [28] (Figure 1C). Therefore, the 2.7-kb element contains the sequences necessary for proper *yellow* expression in the main body of the fly.

The Function of a Discrete *cis*-Regulatory Element Has Diverged between *D. melanogaster* and *D. subobscura*

Since the *D. melanogaster yellow* 5′ sequences were sufficient to control abdominal expression of Yellow,

we hypothesized that evolutionary changes within this region may be responsible for interspecific differences in Yellow expression. To test this hypothesis, we examined the function of orthologous regulatory sequences of the *D. subobscura yellow* gene. Noncoding sequences of the *D. melanogaster* and *D. subobscura yellow* genes share little sequence similarity; however, a number of short, colinear blocks of similar sequence exist that suggest that the organization of the two genes may be conserved [39] (Figure 3A). *Cis*-regulatory regions controlling the expression of Yellow in the abdomen may therefore be located upstream of the transcriptional start site in *D. subobscura*, just as they are in *D. melanogaster*.

No *yellow* mutants of *D. subobscura* were available, so we were unable to test whether the cloned *D. subobscura yellow* sequence contains sufficient regulatory information to fully recapitulate the *D. subobscura* Yellow expression pattern. Some evidence suggests that this sequence contains the entire body enhancer and a small part of the wing enhancer: the most 5′ block of sequence similarity with *D. melanogaster* is located near the boundary between the wing and body enhancers (Figure 3A), and the transformation of the *D. subobscura yellow* gene into *D. melanogaster* resulted in expression identical to endogenous *D. subobscura* expression in the abdomen (Figure 2C), with lower than wild-type levels of expression in the wing (data not shown).

To determine if the 5′ sequence of the *D. subobscura yellow* gene also controls expression in the abdomen, we isolated a region of sequence upstream of the transcriptional start site of *D. subobscura* that is believed to be orthologous to the body enhancer of *D. melanogaster*. A highly conserved 33-bp stretch (94% identical) located 374 bp upstream from the initiation codon in *D. melanogaster* and 497 bp in *D. subobscura* was used as the 3′ end in both constructs (Figure 3A). All available *D. subobscura yellow* sequence upstream of this conserved block was used (1.5 kb total). This sequence was placed into the same Timer reporter gene construct as the *D. melanogaster* 5′ region, it was transformed into *D. melanogaster*, and its expression was analyzed.

The upstream sequence of the *D. subobscura yellow* gene was sufficient to drive the same pattern of reporter expression in the abdomen of *D. melanogaster* as the entire *D. subobscura yellow* transgene. In *D. melanogaster* transformants carrying the *D. subobscura yellow* transgene, high levels of Yellow protein were present throughout the abdominal tergites at all stages examined (Figure 2C; data not shown). Similarly, the *D. subobscura* reporter gene was expressed at very high levels throughout the developing abdominal tergites during late pupal development, as shown by the presence of the green and red Timer isoforms (Figures 3H–3J). Because the expression patterns of the *D. melanogaster* and *D. subobscura* Timer reporter genes in *D. melanogaster* transformants were different, evolution of the *cis*-regulatory sequences controlling abdominal Yellow expression is responsible for the species-specific expression patterns.

Evolution of *trans*-Regulatory Factors Has also Contributed to the Divergence of Yellow Expression

The *D. melanogaster* transformants described above indicated that the transcription factors necessary for the proper expression of the *D. virilis* and *D. subobscura yellow* genes are conserved in *D. melanogaster*. These experiments, however, cannot determine whether the *trans*-regulatory environment necessary for proper expression of the *D. melanogaster yellow* gene is conserved in other species. To test this, we introduced the *D. melanogaster* and *D. virilis yellow* transgenes into *D. virilis*.

The P element-mediated transformation technique commonly used in *D. melanogaster* does not function in *D. virilis* [43]; thus, an alternative transformation method was necessary. A Hermes transposable element [44] carrying a dominant selectable marker [45] has recently been shown to transpose in *D. virilis* embryos [46]. However, germline transformants had not been isolated, the transformation of large pieces of foreign DNA had not been tested, and questions had been raised regarding the stability of a related transposable element (hobo) in *D. virilis* [47]. We found that Hermes transposable elements carrying up to 12 kb of DNA in addition to the transformation marker gene were integrated into the *D. virilis* genome of 3%–6% of the fertile, injected G_0 animals. Furthermore, these transformant lines have been stably maintained in the laboratory for over 18 months (approximately 24 generations).

In order to distinguish between Yellow expression derived from the transgenes and endogenous Yellow expression, we needed to use a *yellow* mutant strain of *D. virilis* as the transformation host. Fortunately, a putative mutation in the *D. virilis yellow* gene had been previously isolated [35]. To determine if this mutant produced any Yellow protein, we compared it to a wild-type strain of *D. virilis* by Western blotting. The Yellow antibody detected a strong band of the expected size in extracts from wild-type flies but did not detect any comparable protein in the mutant extracts (data not shown). Whole-mount immunohistochemical stainings of flies from this mutant strain also failed to detect any Yellow protein (Figure 4A). Therefore, we conclude that the mutant strain used as a transformation host is a protein null mutant of the *yellow* gene.

Immunohistochemical detection of the Yellow protein in *D. virilis* transformants carrying the *D. melanogaster yellow* transgene showed that Yellow was expressed in a pattern that differs from its expression in *D. melanogaster* transformants. Instead of Yellow expression being restricted to a stripe at the posterior of each tergite (Figure 2B), the Yellow protein was present throughout the abdominal tergite at low levels (Figure 4C). This result indicates that the transcription factors controlling expression of *D. melanogaster yellow* have diverged between *D. melanogaster* and *D. virilis*.

However, differences in the *trans*-regulatory environment are not solely responsible for the divergence of *D. melanogaster* and *D. virilis* Yellow expression. Both the *D. virilis* and *D. melanogaster yellow* transgenes were expressed throughout the abdominal tergite in *D. virilis*, but the *D. virilis yellow* transgene produced significantly higher levels of Yellow protein than the *D. melanogaster* transgene (Figures 4B and 4C). This difference in the expression level suggests that the function of the *cis*-regulatory sequences has also evolved, and this observation is consistent with the evidence for *cis*-regulatory evolution from the differential expression of these transgenes in *D. melanogaster*.

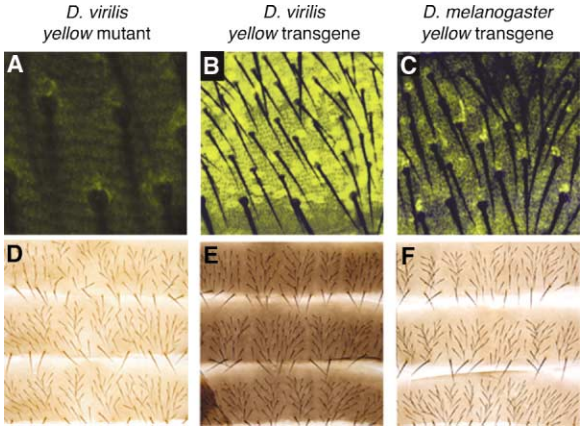

Figure 4. *trans*-Regulatory Factors Controlling the Expression of *D. melanogaster yellow* Have Diverged between *D. melanogaster* and *D. virilis*

(A–C) (A) *D. virilis yellow* mutant flies used as a transformation host do not produce Yellow protein. (The faint yellow color is from autofluorescence of the cuticle). (B) *D. virilis* transformant flies carrying the *D. virilis yellow* gene produce Yellow protein at high levels throughout the abdominal tergite, identical to endogenous *D. virilis* Yellow expression (data not shown, wild-type *D. virilis* expression at an earlier stage shown in Figure 1D). (C) When transformed into *D. virilis*, the *D. melanogaster yellow* gene produces Yellow protein at low levels throughout the abdominal tergite and is not restricted to a posterior stripe as it is in *D. melanogaster* (data not shown, expression at an earlier stage shown in Figure 2B). The Yellow expression shown is in pharate adult abdominal tergites from segment A4; the magnification of (A) is 60×, and the magnification in (B) and (C) is 40×.

(D–F) (D and E) The *D. virilis yellow* transgene fully rescues the pigmentation defects of the *D. virilis yellow* mutant (compare with Figure 1H), (F) whereas the lower level of Yellow expression produced by the *D. melanogaster yellow* transgene results in less melanin production.

D. virilis transformants carrying the *D. melanogaster yellow* gene have reduced melanization relative to transformants carrying the *D. virilis yellow* gene (Figures 4D–4F). This is consistent with the lower levels of Yellow expression activated by the *D. melanogaster yellow* gene (Figures 4A–4C) and the requirement of Yellow for melanin synthesis.

Phenotypic Effects of *yellow* Evolution Depend upon the Genetic Background

Previous experiments have shown that ectopic Yellow expression in *D. melanogaster* is insufficient to induce ectopic melanin patterns [28], and *D. melanogaster* transformants carrying either the *D. subobscura* or *D. virilis yellow* transgene displayed nearly wild-type *D. melanogaster* pigmentation (Figures 2E–2H) despite dramatic changes in Yellow expression (Figures 2A–2D). However, differences in Yellow expression have evolved in concert with differences in pigmentation, suggesting that evolutionary changes in Yellow expression interact with evolutionary changes at other loci to produce species-specific pigmentation.

We identified one wild-type *D. melanogaster* genetic background in which changes in Yellow expression were sufficient to significantly alter melanization. This background carries a genetic modifier that sensitizes the phenotype to the level of Yellow expression. In *yellow* mutant flies homozygous for the modifier, the *D. melanogaster yellow* transgene restored wild-type pigmentation (Figure 5A), whereas the *D. subobscura* and *D. virilis* transgenes increased melanin synthesis throughout the abdominal tergite. The *D. subobscura* transgene (Figure 5B) induced more melanin synthesis than the *D. virilis* transgene (Figure 5C), consistent with the higher levels of Yellow expression produced by this construct (see Figures 2C and 2D).

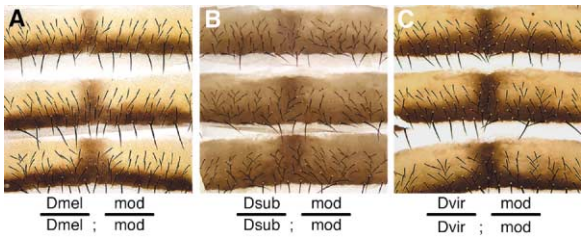

Figure 5. Evolutionary Changes in Yellow Expression Alter Pigmentation in the Presence of a Recessive Modifier

(A–C) In one genetic background, differences in Yellow expression produced by the *D. melanogaster*, *D. subobscura*, and *D. virilis yellow* transgenes (see Figures 2B–2D) result in correlated changes in the adult melanin pattern. (A) The *D. melanogaster yellow* transgene restores wild-type pigmentation in this genetic background, whereas the (B) *D. subobscura* and (C) *D. virilis* transgenes increase melanin production throughout the segment, with more melanin produced by the *D. subobscura* transgene. The sensitivity to Yellow expression of this genetic background is controlled by a recessive modifier on the third chromosome (data not shown). The genotypes of the second and third chromosomes ($2^{nd}/2^{nd}$; $3^{rd}/3^{rd}$) are shown with the following symbols: Dmel, Dsub, and Dvir indicate the *D. melanogaster*, *D. subobscura*, and *D. virilis yellow* transgenes, respectively; mod represents the genetic modifier. All flies are homozygous for a null allele of *D. melanogaster yellow* on the X chromosome.

The two genetic backgrounds shown in Figures 2 and 5 were segregating within a *D. melanogaster yellow* mutant strain that has been maintained in the laboratory for many years; thus, very few genetic differences are expected between the two backgrounds. Genetic mapping indicated that the increased sensitivity to Yellow expression is attributable to a recessive allele(s) on the third chromosome (data not shown). The *ebony* gene is located on the third chromosome (cytological location 93D1) in *D. melanogaster* [48] and encodes a protein that inhibits the ability of the Yellow protein to promote the formation of black melanin [28]. We found that flies heterozygous for the modifier and a loss of function *ebony* allele did not have an *ebony* mutant phenotype, and the *D. subobscura* transgene did not increase melanization in this heterozygous genotype (data not shown). Therefore, we conclude that the modifier is not an allele of *ebony*.

Discussion

We have found that expression of the *yellow* gene, which is required for melanin production, varies among species with differences in abdominal pigmentation and correlates with the distribution of melanin. *Cis*-regulatory changes contribute to the divergence of *yellow* expression among *D. melanogaster*, *D. subobscura*, and *D. virilis*. For *D. melanogaster* and *D. subobscura*, evolutionary changes within an upstream abdominal enhancer are largely responsible for the divergent abdomi-

nal expression patterns. Changes in the distribution of *trans*-regulatory factors have also contributed to the evolution of Yellow expression between *D. melanogaster* and *D. virilis*. Importantly, evolutionary changes in Yellow expression are not sufficient to alter pigmentation in most genetic backgrounds, suggesting that these changes interact with evolutionary changes at other loci to produce differences in pigmentation.

Molecular Mechanisms of Evolutionary Changes in *yellow* Expression

cis-*Regulatory Sequence Evolution*

Transcription of the *D. melanogaster yellow* gene is controlled by multiple, discrete *cis*-regulatory regions (enhancers) [38, 41]. Putative orthologous regions of the *D. subobscura* and *D. virilis yellow* genes have very little sequence similarity to each other or to the *D. melanogaster yellow* enhancers ([39], data not shown). Despite extensive sequence divergence, orthologous upstream regions of the *D. melanogaster* and *D. subobscura yellow* genes both activate transcription in the wing, head, thorax, abdomen, and bristle cells during late stages of pupal development. In the abdomen, expression is initially activated throughout the tergite by both sets of sequences. However, later in development, just prior to eclosion, the *cis*-regulatory element from *D. melanogaster*, but not the element from *D. subobscura*, restricts transcription to a stripe near the posterior side of each tergite. Determining which of the many sequence changes are responsible for this difference in expression will require the analysis of chimeric enhancers and the identification of key transcription factor binding sites.

trans-*Regulatory Evolution*

For a gene to be expressed in a given cell, all of the transcription factors necessary to activate its *cis*-regulatory sequences must be present. *D. subobscura* and *D. virilis yellow* genes were expressed in the same cells of *D. melanogaster* as they are in *D. subobscura* and *D. virilis*, respectively, indicating that transcription factors needed for the activation of these enhancers are present in *D. melanogaster*. However, when the *D. melanogaster* gene was introduced into *D. virilis*, it was expressed in more abdominal cells than it is in *D. melanogaster*, and this expanded expression indicates that the *trans*-regulatory factors controlling expression of *D. melanogaster yellow* are somehow different in *D. virilis*.

At least three potential *trans*-regulatory changes could be responsible for the expanded expression pattern of *D. melanogaster yellow* in *D. virilis*: a positive regulator may be expressed more broadly, a negative regulator may be absent, or changes in the DNA binding specificity of *D. virilis* transcription factors may recognize the *D. melanogaster* binding sites differently. Investigating the actual cause of the difference in *trans*-regulation between these species will first require the identification of the transcriptional regulators of the *D. melanogaster yellow* gene.

The Divergence of Melanin Patterns

Genetic Architecture Underlying Interspecific Pigmentation Differences

One of the major goals of evolutionary biology is to understand the differences in genetic architecture underlying phenotypic differences. The term "genetic architecture" describes the number of genes that contribute to the phenotype, the relative contribution of each of these genes (additive effect), and how these genes work together (epistatic effect). Traditionally, genetic architecture has been investigated by quantitative genetic analysis; however, when the two species being compared are too distantly related to mate or produce fertile offspring, a genetic approach is not possible. None of the species used in this study mate with each other. As an alternative to genetic analysis, we have used transgenic flies carrying heterologous *yellow* genes to examine the phenotypic consequences of evolutionary changes at the *yellow* locus.

yellow expression has evolved in concert with melanin patterns, which strongly suggests that evolutionary changes in Yellow protein expression were a necessary step in melanin pattern divergence. We found that reducing Yellow expression reduces melanin synthesis, but that expanding the spatial domain of Yellow expression usually has little effect on pigmentation [28]. However, we discovered a genetic modifier segregating in a laboratory strain that functions epistatically with expanded Yellow expression. That is, neither the modifier nor increasing Yellow expression alone altered pigmentation, but both genetic changes together produced a novel phenotype. Although the modifier has not yet been cloned, it is likely to be an allele of a gene involved in pigment formation. Many genes function with *yellow* to produce melanin [28, 49], suggesting that there may be other potential modifiers with similar effects.

It is perhaps surprising that the expression of the *D. melanogaster yellow* gene is restricted to a posterior stripe, given that expansion of Yellow protein expression does not significantly alter pigmentation. However, we are comparing species that have been evolving independently for millions of years, and the extant alleles of the *yellow* gene may have had different effects in the original populations in which they arose. For example, a modifier allele such as the one identified in this work may have been fixed in a population, making changes in Yellow expression sufficient to alter pigmentation. Similarly, expression of the Ebony protein, which inhibits the ability of expanded Yellow expression to induce novel patterns of black pigment [28], may have changed since the fixation of extant *yellow* alleles.

An Evolutionary Role for Modifier Genes

Epistatic interactions involving "wild-type" alleles have been reported for other genes (e.g., [50–54]), suggesting that there is an abundance of hidden genetic variation with the potential to contribute to phenotypic variation segregating in both wild populations and laboratory strains. The contribution of these epistatic interactions to phenotypic evolution is a matter of debate, primarily because epistatic allele combinations are expected to be quickly broken up by recombination, especially in large populations. Nonetheless, given the prevalence of interactions among genes and the availability of hidden genetic variation, we find the possibility that epistatic interactions among alleles have contributed significantly to phenotypic evolution both plausible and appealing. (For a more complete discussion of epistasis and evolution, see [55]).

Many studies of melanic polymorphisms in butterfly

and moth populations have found that modifier genes play a major role in determining the phenotype (reviewed in [19]). For example, in the classic evolutionary case of the industrial melanization of the peppered moths (*Biston betularia*), the melanic morph is controlled by a single, dominant allele in the population in which it is most prevalent, but in crosses with nonmelanic moths from another population, the dominant effect of the melanic allele was suppressed by a genetic modifier(s) [56]. Consequently, two moths can carry the same "melanic" allele, but one may not develop the dark coloration.

Melanism has arisen many times in a great variety of taxa [19], and it is likely that similar melanic phenotypes among species may have resulted from different genetic changes. Even within a species, the same pigmentation can result from epistatic interactions among different alleles [57]. We suggest that epistatic interactions may be a common feature of the genetic architecture underlying evolutionary changes in pigmentation.

Conclusions

We have examined the role of the *yellow* gene in the evolution of *Drosophila* melanin patterns, and we found that changes in *yellow* expression have evolved in concert with the divergence of melanin patterns through changes in both *cis*-regulatory sequences and *trans*-regulatory proteins. These changes in Yellow expression were very likely a necessary step in the divergence of pigmentation, but other genetic changes have also evolved that contribute to this phenotype. We identified a modifier segregating within a *D. melanogaster* population that illustrates the phenotypic potential of epistatic interactions among loci. Rapidly evolving melanin patterns of *Drosophila* provide a powerful experimental system for understanding the potential mechanisms of pigmentation evolution in a wide variety of taxa.

Experimental Procedures

Drosophila Strains and Rearing

The wild-type *Drosophila* strains that were used include: *D. melanogaster* (*Canton*^{S}, Carroll lab strain), *D. virilis* (stock #15010-1051.0, Bowling Green, OH stock center), and *D. subobscura* (Arhus, Denmark strain, from G. Gilchrist). The *D. melanogaster yellow* mutant strain (*yellow, white*) used as a transformation host has been maintained in the Carroll lab for many years. The *D. virilis yellow* mutant strain used as a transformation host (*yellow, white*^{a}) was obtained from the Bowling Green, OH stock center (#15010-1051.41). *TM2* and *TM6b* balancer chromosomes carrying the *ebony*^{1} mutant allele, as well as two deficiency stocks missing the *ebony* gene (stock #3340 and #2425, Bloomington, IN stock center) were used in complementation tests. All flies were reared on standard corn meal molasses agar media. *D. melanogaster* and *D. virilis* strains were maintained at 25°C, and *D. subobscura* was raised at 20°C.

Abdominal Cuticle Preparations

Dissection, mounting, and imaging of adult abdomens was as described in [28]. Images of whole flies were captured while they were submerged in 95% ethanol by using a SPOT digital camera (Diagnostic Instruments) connected to a Leica MZ6 microscope. All flies were at least 5 days old, which ensured that pigmentation was fully developed.

Immunohistochemistry

Pupal abdomens representing stages from 72 hr after puparium formation (APF) to pharate adults were immunohistochemically stained with a rabbit polyclonal antibody raised against the *D. melanogaster* Yellow protein as described in [28]. This antibody recognized a protein of the predicted size (approximately 60 kDa) on Western blots of pupal extracts from wild-type *D. melanogaster*, *D. virilis*, and *D. subobscura*, but not in extracts from *yellow* mutant strains of *D. melanogaster* and *D. virilis* (data not shown). *D. subobscura yellow* mutants were not available for analysis. Western blotting was performed as described in [28]. *D. subobscura* and *D. virilis* Yellow proteins are 89% and 82% identical, respectively, to the *D. melanogaster* Yellow protein; thus, it is likely that the affinity of the polyclonal Yellow antibody for all three proteins is similar.

Cloning of the *D. virilis yellow* Gene

P1 clone v10-34 containing the *D. virilis yellow* gene [58] was provided by D. Hartl. DNA from this clone was digested with restriction enzymes and was used for Southern blot analysis. ^{32}P-dATP (NEN) was incorporated into DNA probes by using random prime labeling with the second exon of the *D. melanogaster yellow* gene serving as a template. A 2.6-kb HindIII fragment that hybridized with the probe was gel purified and subcloned into pBlueScript SK+ (Stratagene). In parallel, a *D. virilis* phage genomic library [59] was screened with the same radioactively labeled probe. Four unique phage clones that hybridized with the *D. melanogaster yellow* probe were isolated. Southern blot analysis using probes to the first and second exons of *D. melanogaster yellow* and subcloning of fragments into pBlueScriptSK+ were performed as described above. Each of these subclones was fully sequenced. Alignment of these sequences showed that the six subclones contained overlapping fragments ranging from 2.5 to 4 kb and spanning 11.8 kb in total. Further cloning details are available upon request.

Alignment of the 11.8 kb of *D. virilis* sequence (GenBank Accession Number AY128944) with the sequence of the 7.6-kb *D. melanogaster yellow* gene (GenBank Accession Numbers X04427 and X06481) indicated that the entire coding region of *yellow* was included within this sequence. The sequence also contains 2.6 kb upstream and 850 bp downstream of the translated region. Outside of the two exons, the transcriptional start site, and intron splice junctions, very little sequence similarity was observed. A more extensive analysis of the molecular evolution of *yellow* sequences is underway (P.J.W. and S.B.C., unpublished data).

yellow Transgenes

The 7.6-kb *D. melanogaster* and 7.4-kb *D. subobscura yellow* (GenBank Accession Number Y13909) genes were provided by P. Geyer and C. Segarra, respectively. The overlapping subclones of the *D. virilis yellow* sequence described above were assembled into a single 11.8-kb clone. The *D. subobscura yellow* gene was subcloned into the pCaSpeR vector containing P element ends, and a *white* mini-gene used as a transformation marker. The *D. melanogaster* and *D. virilis yellow* genes were subcloned into the pHer[3xP6 – EGFP] vector [45] containing Hermes transposable element ends and an enhanced Green Fluorescent Protein (EGFP) under the control of multimerized *Pax6* binding sites. The *Pax6*-EGFP construct driving expression of EGFP predominantly in developing and mature eye cells was used as a transformation marker. Cloning details are available upon request.

GFP and Timer Reporter Genes

A derivative of the *hsp70-LacZ* CaSpeR plasmid called RINheXho (expanded polylinker) was used as the starting point for the GFP and Timer reporter vectors. This plasmid contains P element ends used for transposition, a mini-white gene as a transformation marker, and an inactive *hsp70* promoter upstream of the *LacZ* gene. Insertion of enhancer sequences upstream of the *hsp70* promoter activate *LacZ* expression. The coding region of the *LacZ* gene was replaced with the coding region of the S65T variant of the *GFP* gene to generate "RINheXho-GFP" or the E5 mutant of *dsRed* ("Timer") to generate "RINheXho-Timer". pGreen Lantern (GIBCO-BRL) and pTimer-1 (Clontech), respectively, were used as templates to amplify S65T GFP and Timer protein coding regions.

For the *D. melanogaster* "wing-GFP" and "body-GFP" reporters, adjacent HindIII fragments of 0.8 kb and 1.4 kb, respectively (see Figure 3A), were cloned upstream of the promoter in RINheXho-GFP. *D. melanogaster* and *D. subobscura* sequences used to gener-

ate Timer reporter genes (see Figure 3A) were PCR amplified with species-specific forward primers and a common reverse primer containing the *D. melanogaster* version of a highly conserved sequence block just upstream of the transcriptional start site (see Figure 3A). Restriction sites were included at the 5′ end of both primers, and the PCR fragments were cloned upstream of the promoter in RINheXho-Timer. The sequence of all regions of amplified DNA used in these plasmids was confirmed in the final DNA preparation used for injection. Further cloning details are available upon request.

Transformation of *D. melanogaster* and *D. virilis*

Germline transformation of the P element CaSpeR vectors into the Carroll lab *D. melanogaster yellow, white* mutant strain was performed as described in [60]. Transformant flies were identified by the rescue of the *white* mutation with the *white* mini-gene. The germline transformation frequency of the P element vectors used in this study was 5%–10% of fertile G_0 flies.

Transformation of *D. melanogaster* and *D. virilis yellow* mutant strains with the Hermes transposons followed the same procedure, with the following exceptions: pKhsp83Her [61] carrying the Hermes transposase under the control of the *hsp92* promoter was coinjected in place of the P element transposase plasmid, and flies were screened for *Pax6*-EGFP expression instead of rescue of the *white* mutation. Expression of *Pax6*-EGFP in *D. virilis* larvae and adults was significantly weaker than its expression in *D. melanogaster* transformants. Furthermore, the residual eye pigment produced in *D. virilis white*^{a} mutants largely masked EGFP fluorescence in adults. The rescue of the *yellow* mutant phenotype of *D. virilis* with the *yellow* transgene was significantly easier to score than *Pax6*-EGFP expression; thus, the *yellow* gene may provide a better transformation marker for *D. virilis*. Germline transformation frequency of Hermes transposons was about 10% in *D. melanogaster* and 3%–6% in *D. virilis*.

For each transgene, at least three independent insertions were isolated and characterized. Reported phenotypes were shared among all lines.

Imaging GFP and Timer Reporter Gene Expression

Late pupal stages (80 hr APF or older) of transformant flies carrying GFP or Timer reporter genes were carefully dissected from their pupal cases, with the transparent pupal cuticle removed when possible. Flies carrying Timer reporter genes were heat shocked at 37°C for 1–6 hr prior to dissection (see below). Dissected pupae were transferred to a microscope slide containing a drop of water, and a coverslip was applied. Specimens were immediately imaged on a Biorad MRC 1024 confocal microscope.

Maturation of the Timer Protein in *D. melanogaster*

The pattern of Yellow expression during late pupal stages of *D. melanogaster* is temporally dynamic [28]. The same progression of expression pattern was observed with Yellow antibody staining, EGFP reporter genes, and Timer reporter genes; however, specific patterns of Timer protein fluorescence were always observed later relative to other developmental events than EGFP fluorescence or immunohistochemical detection of Yellow protein. Previous reports indicate that the fluorescence of dsRed mutant proteins is significantly slower than EGFP, and that the acquisition of green fluorescence and the maturation of red fluorescence of dsRed mutants are both temperature dependent. At the normal rearing temperature of *D. melanogaster* (25°C), initial fluorescence of a dsRed mutant related to Timer was delayed approximately 15 hr relative to EGFP fluorescence [62]. To get the most accurate reflection of temporal changes in gene expression, and to maximize the fluorescent signal, flies carrying Timer reporter genes were heat shocked at 37°C just prior to dissection. Increasing the length of time of the heat shock (1, 2, 4, and 6 hr were examined) increased the fluorescence intensity approximately linearly (data not shown).

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