

## Reciprocal functions of the *Drosophila* Yellow and Ebony proteins in the development and evolution of pigment patterns

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### SUMMARY

Body coloration affects how animals interact with the environment. In insects, the rapid evolution of black and brown melanin patterns suggests that these are adaptive traits. The developmental and molecular mechanisms that generate these pigment patterns are largely unknown. We demonstrate that the regulation and function of the *yellow* and *ebony* genes in *Drosophila melanogaster* play crucial roles in this process. The Yellow protein is required to produce black melanin, and is expressed in a pattern that correlates with the distribution of this pigment. Conversely, Ebony is required to suppress some melanin formation, and is expressed in cells that will produce both melanized and non-melanized cuticle. Ectopic expression of Ebony inhibits melanin formation, but increasing Yellow expression can overcome this effect. In addition, ectopic expression of Yellow is sufficient to induce melanin

formation, but only in the absence of Ebony. These results suggest that the patterns and levels of Yellow and Ebony expression together determine the pattern and intensity of melanization. Based on their functions in *Drosophila melanogaster*, we propose that changes in the expression of Yellow and/or Ebony may have evolved with melanin patterns. Consistent with our hypothesis, we find that Yellow and Ebony are expressed in complementary spatial patterns that correlate with the formation of an evolutionary novel, male-specific pigment pattern in *Drosophila biarmipes* wings. These findings provide a developmental and genetic framework for understanding the evolution of melanin patterns.

Key words: *yellow*, *ebony*, *tan*, Melanin, Pigment, Abdomen, Evolution, *Drosophila*

### INTRODUCTION

Color patterns are among the most obvious and striking features of animal diversity. The selective forces that affect these patterns, such as predator avoidance, sexual selection and thermotolerance are known (Nijhout, 1991), and in some cases, the genetic basis of intra- and interspecific variation in pigmentation patterns has been examined (for reviews, see Majerus, 1998; Sheppard et al., 1985; Barsh, 1996; Sturm et al., 1998). However, the molecular mechanisms that underlie color pattern development and evolution remain unknown. Before the evolution of pigmentation can be understood, the developmental mechanisms responsible for pigment patterning must be elucidated, which requires answers to the following questions:

What are the functional relationships among genes required for pigmentation?

How do their products determine different pigment types?

What are the molecular mechanisms that control the spatial aspects of pigment patterning?

Melanins, a diverse class of polymerized oxidation products of phenolic precursors, are the most widespread pigments in the biological world, and melanism is one of the most prevalent

modes of color evolution. In animals, melanins are derived from the catecholamine precursors dopa and dopamine, which are synthesized from tyrosine (reviewed by Sturm et al., 1998; Barsh, 1996). Although the biochemical pathways of melanin synthesis are similar throughout animals (Prota, 1992), vertebrates and invertebrates accomplish melanin patterning in their adult bodies by very different mechanisms. Vertebrates synthesize melanin in the neural crest-derived melanocytes in the form of granules, which are exported into the keratinocytes during the formation of skin, fur and hair. Insects, the invertebrates in which pigmentation is best understood, synthesize and secrete melanin precursors throughout their imaginal epidermis during formation of the cuticular exoskeleton (Wright, 1987).

*Drosophila melanogaster* is an excellent model species in which to investigate the regulation of melanin patterns. Some of the enzymatic steps in the melanin synthesis pathway are understood both genetically and biochemically (Wright, 1987). Tyrosine hydroxylase (TH; encoded by the gene *pale*) and Dopa decarboxylase (DDC) convert tyrosine to dopa and dopamine, respectively, which are then processed by a system of Phenol Oxidases (POs) and co-factors to form melanin

during cuticle development. The differential production of pigments among cells generates pigment patterns; therefore, the spatial regulation of the expression or activity of rate-limiting enzymes in this pathway may control pattern formation. Previous work has shown that neither TH nor DDC activity is rate limiting for the production of melanins (True et al., 1999), indicating that other steps required for melanin synthesis control pigment distribution.

*yellow* (*y*) and *ebony* (*e*) are excellent candidates for genes that delimit melanin patterns. In *y* mutants, all black pigment is eliminated, showing that Yellow is required for the production of black melanin (Morgan and Bridges, 1916). Although the biochemical function of Yellow has not been determined, it shares sequence similarity with the recently cloned Dopachrome Conversion Enzyme (Johnson et al., 2001), suggesting that Yellow may have an enzymatic function in melanin synthesis. *e* mutants have the opposite phenotype of *y* mutants: loss of Ebony function increases black pigment (Bridges and Morgan, 1923). *e* encodes the enzyme N- $\beta$ -alanyl dopamine synthetase (NBAD synthetase) that converts dopamine to NBAD, which is subsequently oxidized to produce a tan pigment (Koch et al., 2000; Wright, 1987). This strongly suggests that both an increase in black melanin and a decrease in tan pigment contribute to the dark *e* mutant phenotype.

Although *y* and *e* mutants were discovered many decades ago, their roles in the development of pigment patterns have not been elucidated in detail. Walter and colleagues (Walter et al., 1991) have found that levels of Yellow expression correlate with intensity of black pigment. Additionally, Hovemann et al. (Hovemann et al., 1998) have reported that *e* is expressed during pupal development and was present in the cuticle of young adults. These observations suggest that the spatial regulation of these genes may be important for the proper development of pigment patterns.

We have investigated the genetic interactions between *y* and *e*, and analyzed the regulation and function of these genes during the development of adult pigmentation. We find that adult pigmentation reflects a combination of black and tan pigments, controlled by *y* and *e*, respectively. Yellow is expressed in a temporally dynamic pattern that correlates with black melanin formation, while Ebony is present in cells that produce both melanized and nonmelanized cuticle. Significantly, we find that ectopic expression of Ebony eliminates melanin formation, and, in the absence of Ebony, Yellow promotes the production of black melanin in novel patterns. We conclude that pigment patterns are determined by the combined expression patterns of Yellow and Ebony, and propose that changes in the expression of these genes may have evolved with the interspecific divergence of pigmentation. Consistent with this idea, we find that a novel pigment pattern in another species (*D. biarmipes*) is presaged by complementary spatial patterns of Yellow and Ebony protein expression. These findings demonstrate that the regulation and function of Yellow and Ebony play central roles in both the development and evolution of melanin patterns.

## MATERIALS AND METHODS

### *Drosophila* strains and rearing

*pannier*-GAL4 (*y*<sup>1</sup> *w*<sup>1118</sup>; *P*{*w*+*mW.hs=GawB*}*pnr*<sup>MD237</sup>/*TM3*,

*P*{*w*+*mC=UAS-y.C*}*MC2,Ser*<sup>1</sup>) and UAS-*yellow* (*y*<sup>1</sup> *w*<sup>1118</sup>; *P*{*w*+*mC=UAS-y.C*}*MC1/TM3*, *P*{*UAS-y.C*}*MC2,Ser*<sup>1</sup>) were obtained from G. Morata. UAS-GFP lines (*w*<sup>\*</sup>; *P*{*w*+*mC=UAS-GFP.S65T/T2*} and *w*<sup>\*</sup>; *P*{*w*+*mC=UAS-GFP.S65T/T10*}), *e*<sup>1</sup>, *ry*<sup>12</sup> and *ry*<sup>506</sup> were obtained from the Bloomington, Indiana *Drosophila* Stock Center. In(3R)*e*<sup>AFA</sup> (denoted *e*<sup>AFA</sup>) was obtained from B. Hovemann. The *y* mutants used (*yw*, *yw*<sup>ac</sup>) are standard Carroll lab P-element transformation host strains and *Canton*<sup>S</sup> is a standard Carroll lab wild-type strain. *D. biarmipes* (*rajasekari*) lines 361.0 and 361.3 were obtained from Bowling Green *Drosophila* Stock Center. All flies were reared at 25°C on standard corn meal molasses agar media.

### Dissection, mounting and imaging of adult cuticle

Adult flies (at least 3 days old) were placed in a 10:1 mixture of 95% ethanol and glycerol for 1-3 days. Images of thoraxes were captured from flies submerged in 95% ethanol using a SPOT digital camera (Diagnostic Instruments) connected to a Leica MZ6 microscope. Afterwards, wings were removed at their base and mounted in Hoyer's (Anderson, 1954). For abdominal segments, the head and thorax were removed and the abdominal cuticle cut along lateral midline. Internal soft tissue was removed and the abdominal tergites were mounted in Hoyer's. Wings and abdomens were then baked at 65°C overnight and imaged using a ProgRes 301Z digital video camera (Kontron Electronics) attached to a Zeiss Axiophot microscope. All images taken on the same microscope were captured under identical lighting conditions.

### UAS-Ebony construction

B. Hovemann provided a 1.9 kb *SalI-EcoRI* clone containing the 5' end of the *ebony* cDNA and a 1 kb *EcoRI* clone with the 3' end of the *ebony* cDNA. A 544 bp DNA fragment containing the missing 58 bp of the *ebony* cDNA between the two *EcoRI* sites was PCR amplified from *D. melanogaster Oregon*<sup>R</sup> genomic DNA using the primers 5'-ACGTTCCATTGCTGGTCAAC-3' and 5'-CCAGCACA-TTCCAGATATCG-3'. The sequence of this fragment was compared with the *D. melanogaster* genome sequence (FlyBase, 1999) to ensure that all amino acids were identical. The 58 bp *EcoRI* fragment was assembled with the 5' and 3' clones in the PCR II vector (Invitrogen). The full-length 1.9 kb *ebony* cDNA was then cloned into the pUAST vector (Brand and Perrimon, 1993) as a *NotI-KpnI* fragment and this construct was used for P-element mediated germline transformation of the *yw* strain (Spradling, 1986).

### Antibody production

The full-length cDNA of *ebony*, and a nearly full-length cDNA of *yellow* (missing the first seven amino acids) were cloned into the pGEX 5X-1 vector (Amersham Pharmacia Biotech) in frame with the GST tag. Protein production, inclusion body purification, injection schedule and antibody purification were as described elsewhere (Williams et al., 1995). Briefly, cultures of BL21(DE3)pLysS cells carrying an expression plasmid were induced with 1 mM IPTG. Three hours after induction, the cells were harvested and sonicated to release the protein. The majorities of both GST-Ebony and GST-Yellow proteins were in insoluble inclusion bodies. The insoluble fractions were solubilized by boiling in 5% SDS, diluted, and dialyzed in 1×phosphate-buffered saline (PBS) to remove as much sodium dodecyl sulphate (SDS) as possible. After dialysis, the proteins were diluted to 1 mg/ml in 1×PBS and injected into animals. GST-Ebony was injected into rabbits and GST-Yellow was injected into both rabbits and rats. Solubilized GST-Ebony and GST-Yellow were bound to Actigel (Sterogene) resin and packed into columns. A culture of BL21(DE3)pLysS cells carrying an empty pGEX 5X-1 plasmid was induced, sonicated, bound to Actigel and packed to make a GST column. Serum recovered from all animals was affinity purified using either the GST-Ebony or GST-Yellow column. Recovered rabbit antibodies were then passed over the GST column to remove antibodies that recognized GST and bacterial proteins. Purified

antibodies were diluted to approximately 0.25 mg/ml in 1×PBS, 0.02% sodium azide added and then stored at 4°C. Further details are available upon request.

### Western blotting

Pupae displaying eye pigmentation, but no melanization of the body (approximately 60–75h APF) were homogenized in 125 mM Tris pH 6.8, 6% SDS (100 µl per five pupae), and then centrifuged for 15 minutes. The supernatant was transferred to a new eppendorf tube with an equal volume of 2× Sample Buffer (125 mM Tris pH 6.8, 6% SDS, 0.2% glycerol, 0.25% bromophenol blue, 20 mM dithiothreitol), boiled for 10 minutes, and stored at –80°C. Samples were placed at 95°C for 5 minutes before electrophoresis on 7% polyacrylamide gels (0.5–1 fly equivalent loaded in each lane). Gels were electroblotted onto nitrocellulose membranes using Towbin transfer buffer (192 mM Glycine, 25 mM Tris pH 8, 20% methanol) at 75 mA for 1 hour. Membranes were temporarily stained in 0.02% Ponceau S concentrate (Sigma) to compare the amount of protein loaded per lane, and then blocked in 5% bovine serum albumin (BSA) in TBST (0.02 M Tris pH 7.5, 0.15 M NaCl, 0.03% Tween 20) for 2–12 hours at 4°C. Filters were incubated with primary antibody (1:1000 rabbit anti-Yellow, 1:400 rat anti-Yellow, or 1:400 rabbit anti-Ebony, in TBST 5% BSA) at 4°C overnight and then washed in TBST. Finally, the membranes were transferred to secondary antibodies (Jackson Laboratories) at 1:5000 for donkey anti-rabbit alkaline phosphatase (AP) or 1:2500 for donkey anti-rat AP in PBST + 5% BSA for 2–4 hours at 4°C, washed in TBST, and developed in NBT/BCIP (Roche).

### Immunohistochemistry

For immunohistochemical staining of pupal abdomens and thoraxes, pupae were cut longitudinally near the lateral midline and the dorsal cuticle placed in 1 ml of 1×PBS + 0.3% Triton X-100 (PBST). After gentle shaking to remove non-epidermal cells, formaldehyde was added to a final concentration of 4% to fix the cells and the tissue smoothly rocked at room temperature for 40 minutes. Methylene Blue (50 µl of 0.5%) was added to stain the cells and rocking continued for 5 minutes. Any remaining fat cells, muscles and the pupal membrane were then removed, and samples were blocked in PBST + 3% BSA for at least 1 hour. Samples were then incubated with primary antibodies (rabbit anti-Yellow, rabbit anti-Ebony and/or rat anti-Yellow) at 1:200 in PBST + BSA overnight, followed by four washes for 15 minutes each in PBST and incubation with secondary antibodies at a concentration of 1:200 for at least 4 hours. Secondary antibodies conjugated with FITC, Cy3 or biotin were used to detect specifically primary antibodies generated in rabbits or rats (Jackson Laboratories). Samples were then washed again four times for 15 minutes each in PBST with TOPRO (Molecular Probes) included at 1:2000 in the first three washes. Samples incubated with a biotin-conjugated secondary antibody were subsequently incubated in 1:200 streptavidin conjugated to FITC (Jackson Labs) for 2 hours at 4°C, and then washed in PBST. All steps after fixation were performed at 4°C. Samples were mounted in Vectashield (Vector Labs) and imaged on a Biorad MRC 1024 confocal microscope.

For immunohistochemical staining of pupal wings, the wings were excised in PBS and flattened in fixative under a coverslip for 5–10 minutes, and then placed in a glass scintillation vial containing 4% formaldehyde in PBST for 30–60 minutes on ice. Halfway through fixation, specimens were sonicated for 30–60 seconds in a Branson 200 jewelry cleaner. Wings were then moved to PBST + BSA and processed as described for pupal body staining.

The age of pupae used for immunohistochemistry was estimated based on eye color and progression of melanization as described in Ashburner (Ashburner, 1989). Therefore, all ages given are approximations and are expected to be correct within about 2–3 hours.

### Specificity of Yellow and Ebony antibodies

Western blotting of protein extracts from wild-type and mutant tissues

was employed to test the specificity of the antibodies produced above. Each antibody recognized a protein that was the predicted size of each respective antigen in wild type *D. melanogaster* and *D. biarmipes* flies, and these full-length proteins were absent in the respective genetic null mutants of *D. melanogaster* (see Fig. 2). Immunohistochemical staining was performed to confirm that the antibodies specifically recognized Yellow and Ebony proteins in vivo. To summarize briefly, the Yellow antibody did not produce any signal in *y* null mutants (see Fig. 3A). Similarly, during the mid-late pupal development, Ebony expression detected in wild-type flies was absent in *e<sup>ΔFA</sup>* mutants (see Fig. 4A,B). In pharate adults, however, the Ebony antibody produced a similar signal in both wild-type and mutant animals (see Fig. 4C; data not shown). The *e<sup>ΔFA</sup>* allele is a genetic null mutant for pigmentation, but still produces truncated transcripts of *e* (Hovemann et al., 1998). In the absence of the Ebony antibody, no fluorescent signal was observed in animals of either genotype, suggesting that the polyclonal antibody recognized truncated Ebony proteins in *e<sup>ΔFA</sup>* flies. Yellow and Ebony antibodies were also found to specifically detect ectopically expressed Yellow or Ebony proteins, respectively (see Fig. 3E,F).

## RESULTS

### *yellow* and *ebony* regulate pigment patterns in *D. melanogaster*

To study the functions of *y* and *e* in pigment patterning, we focused on the most dramatic melanin pattern in *D. melanogaster* adults, the dark pigment stripe near the posterior edge of each abdominal tergite (Fig. 1A, arrowhead). In *y* mutants, the appearance of this stripe changed from black to brown, but the pattern remained the same (Fig. 1B). Similarly, in an *e* mutant, the posterior stripe remained distinct, but the cuticle anterior to the stripe was darker than in the wild type (Fig. 1C). Flies that lacked function of both genes displayed a phenotype different from either of the single mutants: the posterior stripe was no longer apparent and the entire tergite assumed a brown color (Fig. 1D). The disappearance of the distinct stripe in the double mutant shows that *y* and *e* are each required for the formation of this pigment pattern.

These genes are also required for proper pigmentation of other structures. Phenotypes of *y* and *e* single and double mutants showed that in both the thorax and the wing, endogenous Ebony expression suppresses black pigment patterns that required the Yellow protein. In both structures, loss of *y* function altered the overall pallor of the cuticle relative to wild type, but did not show any alteration in the pigment pattern (Fig. 1E,F,I,J). In *e* mutants, however, a dramatic pigment pattern, referred to as the ‘trident’, formed in the thorax and pigment surrounded the wing veins (Fig. 1G,K). Flies lacking both Yellow and Ebony retained these patterns, but black melanin appeared to be absent (Fig. 1H,L).

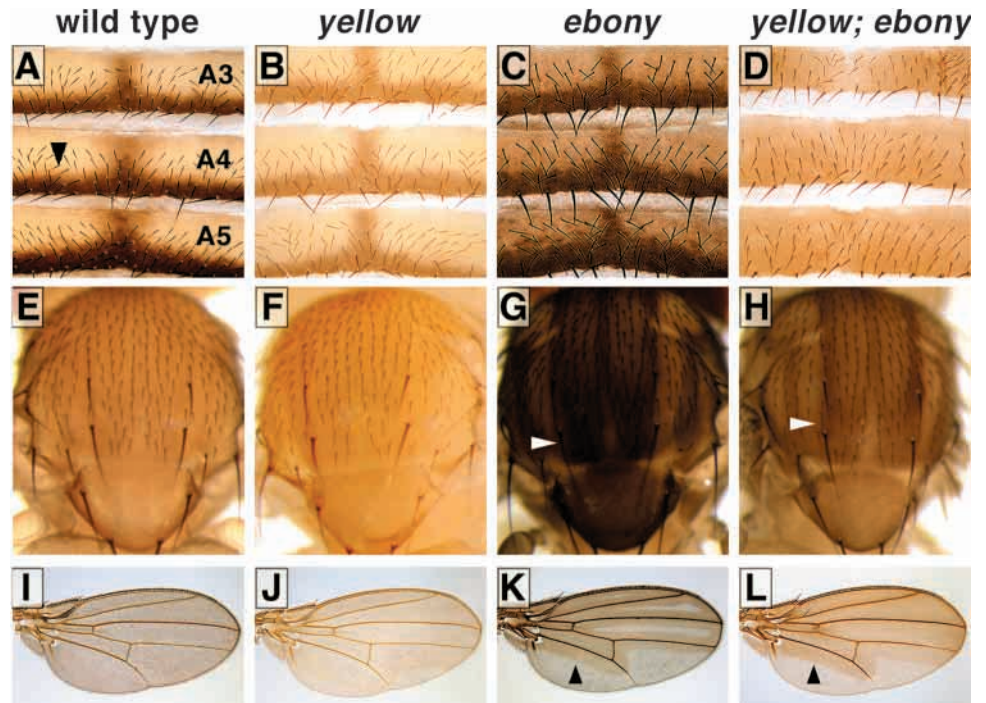
The requirement of *y* and *e* to promote and inhibit black pigment, respectively, strongly suggests that adult pigment patterns may reflect spatially regulated expression of these genes. Therefore, we generated antibodies that specifically recognize the Yellow and Ebony proteins (Fig. 2; also see Materials and Methods) and determined the spatiotemporal distribution of these proteins.

### Spatial distribution of Yellow protein correlates with black pigment

It has been shown that *y* function is required during mid to late pupal stages for proper pigmentation of the adult (Nash, 1976),



**Fig. 1.** *yellow* and *ebony* are required for proper pigment patterning in *D. melanogaster*. (A) Wild type *D. melanogaster* females produce a stripe of dark pigment (arrowhead) near the posterior edge of abdominal segments A2-A6. (B) In *yellow* null mutants, black melanin is lost within the stripe, leaving a brown pigment, and cuticle anterior to the stripe has a tan appearance relative to wild type. (C) *ebony*<sup>1</sup> mutants retain a distinct pigment stripe and the cuticle anterior to the stripe is much darker than wild type. (D) *yellow; ebony*<sup>1</sup> double mutants have brown pigment throughout the abdomen and the stripe is no longer distinct. Similar changes in pigmentation are observed in other structures. Wild-type flies are a uniform color in the thorax (E) and wings (I). In *yellow* mutants, these structures become tan (F,J). In *ebony*<sup>1</sup> mutants, the thorax (G) and wings (K) are more darkly pigmented, and new pigment patterns are visible (arrowheads). *yellow; ebony* double mutants also show these patterns, but the black pigment is absent, and they consist of two shades of brown pigment (H,L, arrowheads). In all panels, anterior is upwards and dorsal cuticle is shown. Abdominal segments A3-A5 are shown in A-D.



and the Yellow protein has been observed everywhere that black pigment forms in the adult (Walter et al., 1991). Furthermore, the levels of Yellow expression were found to correlate with the intensity of black pigment (Walter et al., 1991). The resolution of these findings, however, was limited by visualization of Yellow distribution only in tissue cross-sections. In order to characterize Yellow expression more fully, we used a whole-mount immunohistochemical staining protocol (Kopp and Duncan, 2000) that allowed us to examine protein expression throughout the entire developing dorsal epidermis.

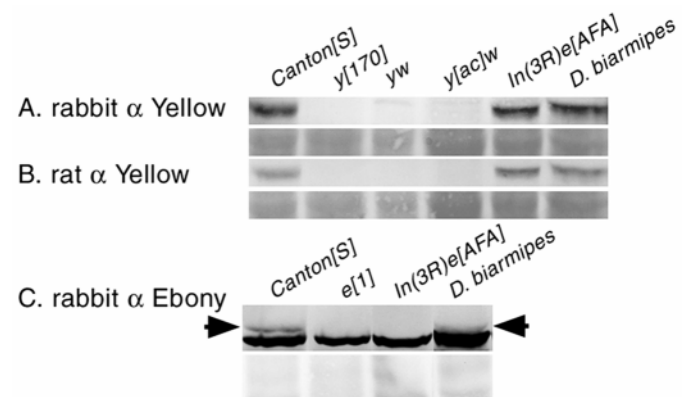
In the abdomen, Yellow protein was present in cells throughout each of the abdominal tergites from approximately 60-72 hours after pupal formation (APF) (see A3 segment in Fig. 3B). Later, starting at approximately 72 hours APF, Yellow was predominantly restricted to cells that produce the abdominal pigment stripe (see A4 segment in Fig. 3B). Temporal refinement of Yellow expression began in the

anterior-most segment and progressed posteriorly (Fig. 3B), consistent with the polar wave of development of other abdominal structures (Bainbridge and Bownes, 1981). In the A5 abdominal segment, Yellow expression was sexually dimorphic with high levels throughout the segment in males, but not in females (data not shown). This expression correlates with high levels of male-specific abdominal pigment in the A5 segment of adults. Yellow was also expressed in a cell associated with each adult mechanosensory bristle and throughout the developing wing blade (Fig. 3C, arrow; data not shown), consistent with the genetic requirement of *y* for normal pigmentation of these structures. Finally, Yellow protein was present in the cells of the thorax that form the black 'trident' in *e* mutants (Fig. 3C, also see Fig. 1G).

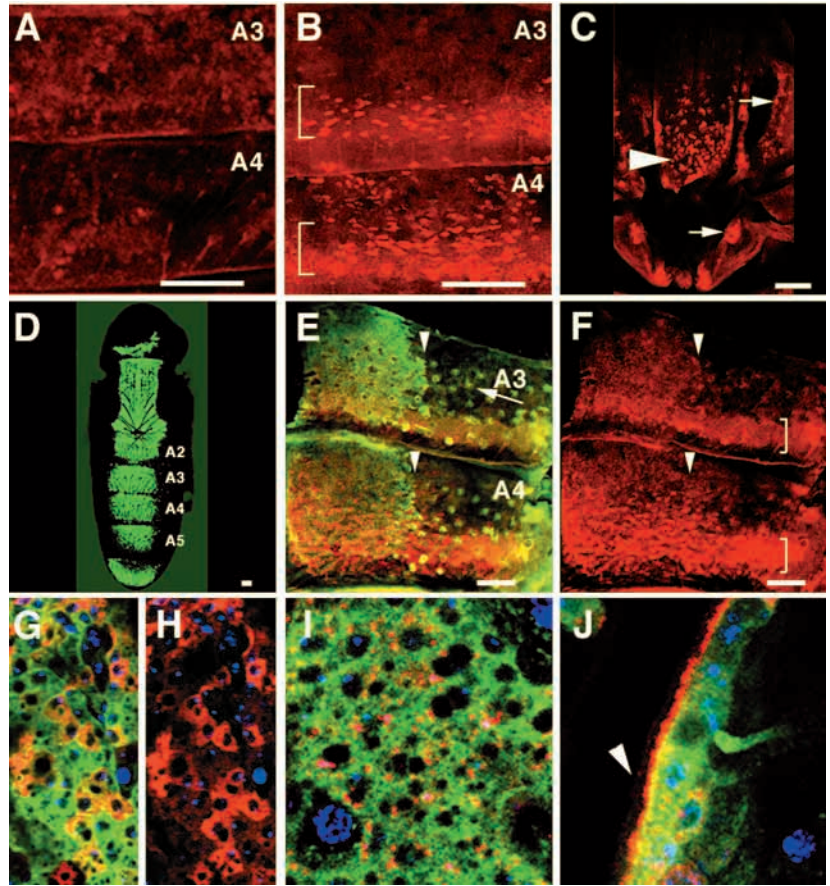
### Post-transcriptional regulation and processing affect the distribution of the Yellow protein

In addition to determining the spatial distribution of the Yellow

**Fig. 2.** Antibodies specific for the *Drosophila* Yellow and Ebony proteins. Western blots of ~60-72 hour pupal protein extracts. Antibodies to the Yellow protein produced in rabbits (A) and rats (B) recognize a 60 kDa protein in protein extracts from animals with a wild-type *y* gene (*Canton<sup>S</sup>*, *e<sup>AFA</sup>*), but not from *y* mutants (*y<sup>170</sup>*, *yw*, *yw<sup>ac</sup>*). Both antibodies also recognize a similar size protein in *D. biarmipes*. (C) Antibodies raised in rabbits against the Ebony protein recognize a 94 kDa protein (arrowheads) in wild-type animals (*Canton<sup>S</sup>*), but not *e* mutants (*e<sup>1</sup>*, *e<sup>AFA</sup>*). Several smaller proteins in both wild-type and *e* mutant flies are also detected (data not shown). A protein of approximately 94 kDa is recognized in extracts from *D. biarmipes*. A strong band just below the presumptive Ebony band also appears in both wild-type and *e* mutant extracts. (A-C) The panel below each blot indicates relative protein loading.



**Fig. 3.** The spatial pattern and subcellular distribution of the Yellow protein is temporally dynamic. The final distribution of Yellow in late pupal stages correlates with the location and intensity of black melanin in the adult. (A) Immunohistochemical staining with the anti-Yellow antibody does not recognize any proteins in a *yellow* mutant. Abdominal segments A3 and A4 from a pupa 72 hours after puparium formation (APF) are shown. (B) Wild-type (*Canton<sup>S</sup>*) pupa approximately the same age as in A. In the A3 segment, Yellow protein is present almost exclusively in the cells that secrete the pigment in the stripe (bracket). Yellow is expressed in this pattern in all segments at later developmental stages. In the A4 segment, Yellow protein is present in cells that underlie the future pigment stripe (bracket), as well as in more anterior cells that produce significantly less black melanin. During earlier pupal stages, the distribution of Yellow in all segments resembles the A4 segment shown. The change in the spatial distribution of Yellow protein occurs first in A2 and progresses posteriorly to A6. The pupa shown in B has undergone this refinement in A3, but not yet in A4. (C) In the thorax, at approximately 80 hours APF, Yellow protein is present in cells that produce the thoracic pigment pattern in *ebony* mutants (arrowhead; see Fig. 1C). Additionally, Yellow is expressed in a cell associated with each mechanosensory bristle (arrows). (D) Expression of UAS-GFP (green) shows that the *pannier*-Gal4 driver is expressed in dorsal cells along the length of the fly. (E-I) Co-expression of UAS-Yellow and UAS-Ebony activated by *pannier*-Gal4. (E-H) Ebony (green) is present in all cells within the *pannier*-Gal4 expression domain, whereas, Yellow protein (red) is only present in a subset of these cells. (E,F) Abdominal segments A3 and A4 are shown with the dorsal midline at the left edge, and the lateral midline at the right edge. Arrowhead indicates the edge of the *pannier*-Gal4 expression. Endogenous Yellow protein underlying the pigment stripes (brackets) and endogenous Ebony expression (arrow) are also detected. (G,H) Initially, ectopic Yellow is present in the cytoplasm of a subset of cells in which it is transcribed. The presence of ectopic Ebony protein indicates transcriptional activation of UAS by *pannier*-Gal4. TOPRO staining (blue) shows the location of both epidermal nuclei and the larger bristle cell nuclei. Later in development, cytoplasmic expression of Yellow forms foci within the cell (data not shown) which are subsequently exported and evenly distributed among neighboring cells (I). The transition of the Yellow protein from cytoplasmic to extracellular occurs in an anterior-to-posterior wave, similar to the change in spatial expression pattern. In the A4 segment shown in F, Yellow expression is still predominantly cytoplasmic near the posterior of the segment, but becomes diffuse foci in the more anterior cells and in the A3 segment. (J) An optical cross section shows that after it is exported, Yellow protein (red) becomes evenly distributed above the apical side of epidermal cells that directly underlie the developing cuticle (arrowhead). TOPRO staining (blue) and Ebony expression (green) show the nuclear and cytoplasmic boundaries, respectively. Apical is towards the left. Scale bars: in A-F, 100  $\mu$ m.



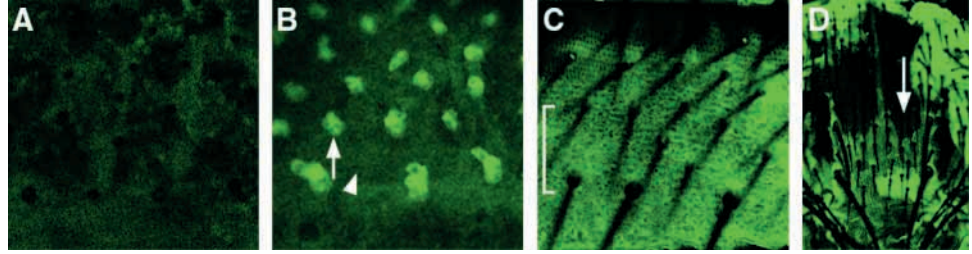
protein, we compared the expression levels of Yellow among neighboring cells, as well as determined the subcellular localization and distribution of the protein. Unexpectedly, the amount of Yellow protein varied among cells fated to produce black pigment (Fig. 3B). To determine if this variability was due to differences in transcriptional or post-transcriptional regulation of  $\gamma$  among cells, we ectopically expressed Yellow in a broad stripe along the dorsal midline of developing flies using the *pannier*-Gal4 driver (Fig. 3D) and monitored the distribution of the Yellow protein. Similar to endogenous Yellow, the levels of ectopically expressed protein were variable among cells (Fig. 3B,E,H). Control experiments using a UAS-GFP reporter indicated that the Gal4 activator protein was produced at equivalent levels in all cells within the *pannier* expression domain (data not shown). Furthermore, we co-expressed ectopic Yellow and Ebony proteins under identical transcriptional control and stained for both antigens

simultaneously. Ebony protein was detected uniformly in all cells that expressed the transcriptional activator, indicating that the non-uniform Yellow staining was not a technical artifact (Fig. 3E-H). Therefore, the observed differences in Yellow protein levels are due to differences in post-transcriptional regulation between cells.

How can non-uniform expression of Yellow lead to a uniform distribution of pigment? Biochemical studies have shown that the Yellow protein is processed, secreted and accumulates on the apical surface of the cell (Kornezos and Chia, 1992). Once outside the cell, the Yellow protein may move to neighboring cells, as suggested by the ability of Yellow to rescue pigmentation of  $\gamma$  mutant cells a few diameters from where it is expressed (Hannah, 1953). Yellow is thought to then become incorporated into the developing cuticle, where it affects pigmentation development approximately 24 hours later (Kornezos and Chia, 1992).



**Fig. 4.** Ebony protein is expressed widely and does not correlate with a single pigment. (A) Immunohistochemical staining of *e<sup>ΔFA</sup>* with the Ebony antibody does not detect any staining at approximately 72 hours APF. In pharate adults, a weak signal is produced in epidermal cells (data not shown). (B) From approximately 72 to 90 hours APF, Ebony protein (green) is expressed in cells associated with mechanosensory bristles (arrow), but not in epidermal cells (arrowhead). This expression may not function in pigmentation because *e* mutants have wild-type bristle color. (C) Beginning at approximately 90 hours APF, low levels of Ebony protein (green) are present in all epidermal cells of each abdominal segment. Bracket indicates the future location of the pigment stripe and staining of cells near the top of the panel is in a different focal plane. (D) In the thorax, highest levels of Ebony expression are in epidermal cells that produce the ‘trident’ in *e* mutants (arrow). The strong staining seen near the top of the panel is background signal from underlying tissues.



Immunolocalization of the Yellow protein presented here supports and extends each of these observations. Yellow protein initially accumulated in the cytoplasm of a subset of cells in which it was transcribed (Fig. 3B) and later resolved into punctate foci within each of these cells (data not shown), consistent with post-translational processing of the protein. Subsequently, these foci were distributed on the apical surfaces of adjacent cells (Fig. 3I,J), suggesting that the protein was exported and translocated. At the time Yellow apparently incorporated into the cuticle, the protein was evenly distributed over all cells that underlie black melanin patterns (data not shown).

#### **Ebony expression is not spatially regulated in the abdomen, despite its requirement for pigment patterning**

Our genetic experiments suggest that the spatial regulation of *e* expression may also be necessary for patterning the abdominal pigment stripe. To test this hypothesis, we examined the distribution of the Ebony protein in abdominal epidermal cells during pupal development. During most of pupal development, the Ebony protein was not detected in these cells (Fig. 4B, arrowhead). In pharate adults, however, just before eclosion, low levels of the Ebony protein were present in the cytoplasm of most epidermal cells. All cells that secrete the abdominal tergites produced the same amount of Ebony protein, regardless of their position within the segment (Fig. 4C). Therefore, in the pupal abdomen, Ebony expression is not spatially regulated and does not correlate with a single pigment type.

*e* function is also required for proper pigmentation of the thorax and wings, and in *e* mutants, ectopic pigmentation was observed in these structures (Fig. 1G,K). In the thorax, Ebony protein expression was highest in epidermal cells that underlie the ‘trident’ pigment pattern in *e* mutant adults (Fig. 4D; Fig. 1G), whereas in the wing, Ebony was equally distributed across all cells (data not shown). Spatial regulation of Ebony in the thorax and not the wing, suggests that the suppression of black pigment in these structures may occur through different molecular mechanisms (True et al., 1999).

#### **Changing Yellow and Ebony protein expression alters pigment patterns**

Because Yellow and Ebony are required to delimit pigment patterns, we hypothesized that changing the expression level

and/or spatial distribution of these proteins may be sufficient to alter pigmentation. To test this hypothesis, we used a *pannier*-Gal4 driver to activate UAS sequences controlling expression of Yellow and/or Ebony in a defined subset of pupal epidermal cells (Fig. 3D). The level of expression induced in these cells is similar to level of endogenous Yellow protein and significantly higher than the level of Ebony protein normally present (Fig. 3E).

Ectopic expression of Yellow protein did not dramatically change the pigmentation phenotype. In the thorax, pigmentation was slightly darkened compared with that of wild-type flies (Fig. 1E, Fig. 5A). Similarly, the effects of ectopic yellow expression in the abdomen were subtle. The color intensity and the anteroposterior width of the abdominal pigment bands were mildly increased within the *pannier* expression domain (Fig. 1A, Fig. 5B, arrowheads), and the lateral width of the pigment along the dorsal midline also increased (Fig. 1A, Fig. 5B, arrow). However, in many cells, such as those anterior to the pigment stripe, Yellow expression was not sufficient to induce black pigment.

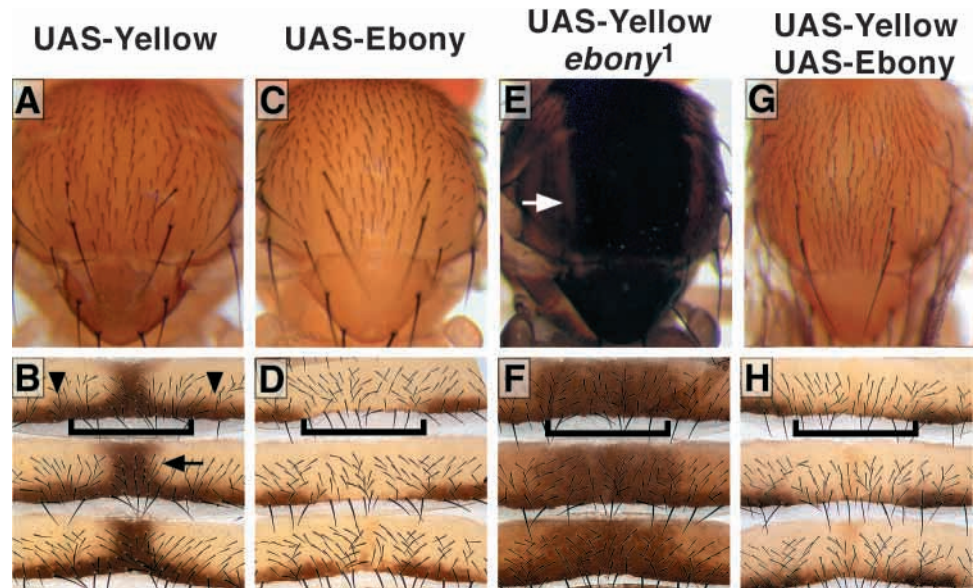
Conversely, we found that ectopic expression of Ebony was sufficient to suppress black pigment formation in all epidermal cells tested. In the thorax, ectopic expression of Ebony subtly lightened the cuticle relative to wild-type (Fig. 1E, Fig. 5C). In the abdomen, ectopic Ebony expression led to a striking inhibition of melanin formation, which was replaced with tan pigment (Fig. 1A, Fig. 5D).

Because the Ebony protein inhibits melanin formation, we hypothesized that endogenous Ebony may inhibit ectopic Yellow protein from inducing the formation of black pigment. To test this possibility, we ectopically expressed Yellow in an *e* mutant background. In the absence of the Ebony protein, ectopic expression of Yellow caused a dramatic increase in black pigment in the thorax and in the abdomen (Fig. 5E,F), indicating that the Ebony protein prevents Yellow from promoting black pigment formation.

In wild-type flies, however, black pigment is produced by cells that express high levels of Yellow and low levels of Ebony, suggesting that at high expression levels, the Yellow protein may override the inhibitory effects of Ebony. To test this hypothesis, we ectopically expressed both proteins together. In the abdomen, ectopic expression of both Yellow and Ebony proteins induced the formation of more black melanin than the did ectopic expression of Ebony alone (compare Fig. 5D with 5H), although the wild-type level of pigmentation was not fully restored. Co-expression of ectopic

**Fig. 5.** Changing Yellow and Ebony expression is sufficient to alter pigment patterns. (A) Ectopic expression of Yellow results in a subtle increase of black pigment in the thorax relative to wild type. (B) In the abdomen, this expression causes a slight widening of the pigment stripe (arrowhead indicates the border between wild-type and ectopic pigmentation), as well as the dorsal midline pigment (arrow). (C) Ectopic expression of Ebony in the same cells results in a tanning of the thorax and a removal of melanin in the abdominal stripe (D). (E) In *ebony*<sup>1</sup> mutants, ectopic expression of Yellow induces black pigment in the thorax (arrow) and the abdomen (F).

(G,H) Co-expression of Yellow and Ebony results in a phenotype more similar to wild type than does ectopic expression of either protein alone. (B,D,F,H) Brackets indicate the approximate boundaries of *pannier*-Gal4 expression.



Yellow and Ebony in the thorax also resulted in a phenotype more similar to wild-type than to the phenotype caused by ectopic expression of Ebony alone (Fig. 1E, Fig. 5C,G). Therefore, Ebony is not fully epistatic to Yellow, and the ratio of Yellow and Ebony proteins within a cell may be critical for determining pigmentation intensity.

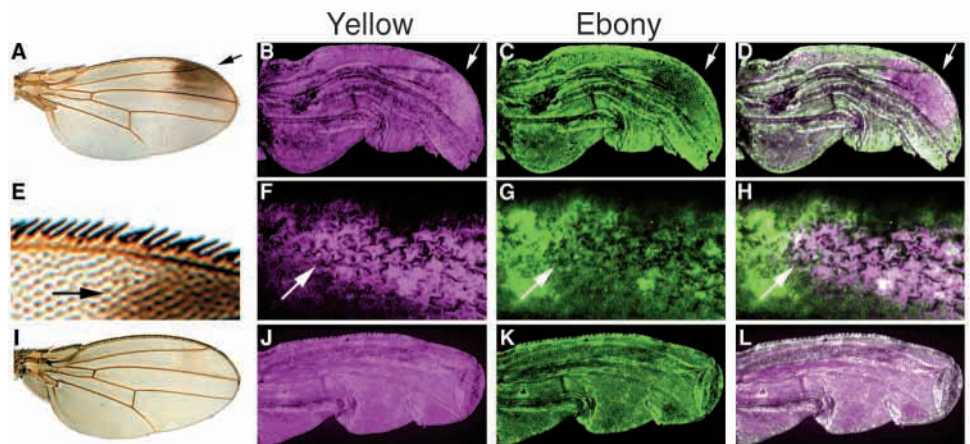
### Changes in Yellow and Ebony expression correlate with a novel pigmentation pattern

Based on the functions of *y* and *e* in *D. melanogaster*, we hypothesized that evolutionary changes in expression of these genes may be involved in the divergence of pigment patterns between *Drosophila* species. We tested this by examining the distributions of Yellow and Ebony proteins in a species with a novel pigment pattern. *D. biarmipes* (also called *D. rajasakari*) is a member of the *melanogaster* species group and bears a male-

specific spot of black pigment on the wing that *D. melanogaster* lacks (Fig. 1I, Fig. 6A,E,I). If changes in *y* and/or *e* expression were involved in the evolution of this pigment pattern, then the distribution of these proteins should correlate with the wing spot in a manner consistent with their functions in *D. melanogaster*.

We found that the Yellow and Ebony proteins were present in the developing wings of *D. biarmipes* in complementary patterns that correlate with the wing pigment spot. In males, high levels of Yellow were expressed in an area of the wing that produces the pigment spot (Fig. 6B,D,F,H). By contrast, Ebony protein levels in these cells were lower than in surrounding cells (Fig. 6C,D,G,H). In females, however, Yellow and Ebony were expressed evenly throughout the wing (Fig. 6J-L), suggesting that females do not produce the pigment spot because Yellow and/or Ebony expression are not modulated in these cells.

**Fig. 6.** Complementary patterns of Yellow and Ebony expression correlate with the formation of a novel, male-specific, black melanin pattern in *D. biarmipes* wings. (A) A spot of black melanin (arrow) is present in the wings of *D. biarmipes* males. (B,D) Yellow protein (purple) is expressed at higher levels in the cells that produce this spot (arrow) than in the surrounding wing. (C,D) Ebony expression (green) is lower in these cells (arrow) than in the rest of the wing. (E-H) The boundaries between expression levels of Yellow and Ebony coincide (F-H, arrows) and correlate with the boundary of pigment in adult wings (E, arrow). (I) *D.*



*biarmipes* females, typically do not produce a pigment spot in the wing, and both Yellow (J,L) and Ebony (K,L) proteins are uniform throughout the wing in most females. In some adult females, a small, faint pigment spot is observed (data not shown). Consistent with this phenotypic variation, a few cells expressing higher levels of Yellow are present in a small proportion of the female pupal wings (data not shown).



## DISCUSSION

We have shown that the *Drosophila* *y* and *e* genes play critical, reciprocal roles in the formation of melanin patterns. Yellow protein is required for the production of black melanin, Ebony protein is required to produce tan pigment, and in the absence of both genes, a brown pigment remains. We find that the distribution of the Yellow protein correlates with the pattern and intensity of black melanization in the adult, and that changes in this expression pattern alter melanin patterns in the absence of Ebony. Surprisingly, we find that the Ebony protein is not spatially regulated, despite its function in pigment patterning. Its low level, uniform expression is necessary for proper pigmentation, and differences in the level of Ebony expression among cells affects pigment patterns. Finally, we have shown that evolutionary changes in Yellow and Ebony expression correlate with the development of a novel melanin pattern.

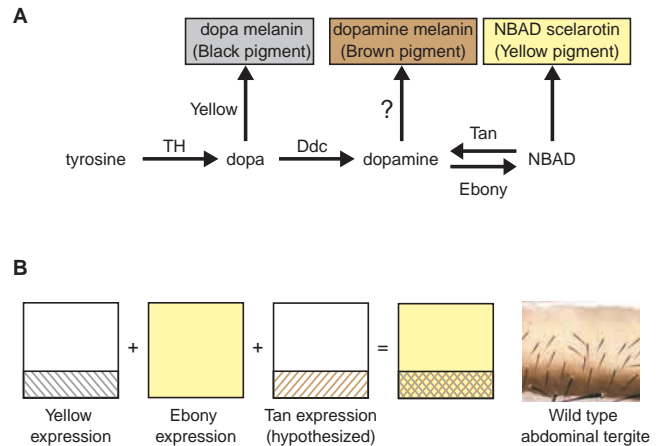
Based on our findings, we propose a new model for the genetic, biochemical and molecular mechanisms of pigment formation and its spatial patterning (Fig. 7). Our model integrates the data presented here with the existing picture of melanin biosynthesis (reviewed by Wright, 1987), and incorporates additional unpublished observations. Previously unresolved issues, such as the relationships between the phenotypes of pigmentation mutants, the biochemical synthesis of different pigments, and the spatial regulation of pigment production, are addressed.

### Pigment production and spatial patterning in *Drosophila*

Our model of the *Drosophila* melanin biosynthesis pathway is illustrated in Fig. 7A. The conversion of tyrosine to dopa by TH and the subsequent conversion of dopa to dopamine by DDC are well supported by genetic and biochemical data (Wright, 1987). Similarly, the functions of the Ebony and Tan proteins in a reversible reaction between dopamine and NBAD have also been biochemically established (Wright, 1987). We propose that there are three branches that emanate from a central pathway, and that each branch produces a distinct pigment color. First, dopa is converted into black dopa-melanin by a branch that depends on Yellow activity. Second, dopamine is converted to NBAD via Ebony function and then back to dopamine by the Tan protein before polymerization into brown dopamine-melanin. Third, tan pigment is produced from the oxidation of NBAD, the production of which requires Ebony. Final pigmentation of the adult reflects the combined spatial distributions of dopa-melanin (black), dopamine-melanin (brown), and an NBAD polymer (tan). We discuss the evidence supporting our assertions that both dopa and dopamine-melanin contribute independently to dark pigmentation, that Yellow is required for the production of dopa-melanin and that the indirect production of dopamine, which requires both Ebony and Tan proteins, is a necessary step in the production of dopamine-melanin.

### Separate contributions of dopa and dopamine to dark pigmentation

Previous work in *D. melanogaster* suggested that its predominant pigment was dopamine-melanin (Wright, 1987; Walter et al., 1991). In numerous other insects, however, dopa-melanin is thought to be equally important (Nijhout, 1991; Johnson et al., 2001), and we propose that dopa-melanin does,



**Fig. 7.** Proposed biochemical and molecular mechanisms for pigment synthesis and patterning. (A) Dopa-melanin, dopamine-melanin and NBAD scelerotin are products of discrete branches of a common biochemical pathway. Yellow, Tan and Ebony proteins are rate-limiting enzymatic steps in the formation of black, brown and tan pigments, respectively. The '?' indicates the activity of an unknown gene that regulates pigment patterns in the absence of *tan* (P. J. W., unpublished). (B) Development of the abdominal pigment stripe in *D. melanogaster* requires spatial regulation of Yellow, Ebony and Tan. Boxes represent a section of abdominal tergite as shown in the far right panel. Yellow expression is gray, Ebony expression is yellow and our prediction of Tan expression is brown. The combined action of these patterns is to induce the formation of black melanin in the stripe where Yellow is expressed, tan pigment anterior to the stripe where Ebony is expressed alone, and brown melanin in the stripe where both Ebony and Tan are present. The combination of black and brown melanins produces the final appearance of the stripe.

in fact, contribute significantly to the pigmentation of *D. melanogaster*. Cells that lack the function of the DDC protein are unable to produce dopamine. Nevertheless, in *ddc* mutant clones, and in *ddc* mutant flies that escape lethality, some cells still produce a gray pigment (True et al., 1999; Wright et al., 1976). Because dopamine-melanin cannot be produced in the absence of the DDC protein, we infer that this gray pigment is dopa-melanin.

Consistent with this inference, spontaneous oxidation of dopa produces a gray (black) pigment, whereas oxidation of dopamine and NBAD produces brown and tan pigments, respectively (J. R. T., unpublished). We have also found that incubation of dopa, dopamine or NBAD with phenoloxidase, an enzyme required *in vivo* for polymerization of melanins, produces the same three distinct colors of pigment (True et al., 2001) (J. R. T., unpublished). Based on these observations, we propose that black, brown and tan pigments observed in adult flies reflect the production of dopa-melanin, dopamine-melanin and an NBAD polymer, respectively, by three branches of the melanin synthesis pathway.

### Yellow may function in dopa-melanin production

Yellow is required for the formation of black pigment that is likely to be dopa-melanin, and shares sequence similarity with an enzyme that catalyzes a reaction necessary for the conversion of dopa to dopa-melanin in the mosquito (Johnson et al., 2001). These observations suggest that Yellow may also



encode an enzyme that is needed for the production of dopa-melanin. Biochemical analysis of the Yellow protein is necessary to test this hypothesis.

#### Ebony and Tan affect dopamine-melanin production

Perhaps the most intriguing relationship within the melanin synthesis pathway is the apparent opposing functions of the Ebony and Tan proteins. *e* mutants do not produce NBAD or tan pigment, and *tan* (*t*) mutants lack brown pigment and have reduced dopamine levels (Wright, 1987). The loss of brown pigment in *t* mutants suggests that the conversion of dopamine into NBAD and then back to dopamine before polymerization is a necessary step in the production of brown dopamine-melanin. *e*, *t* and *e;t* mutant phenotypes also suggest a requirement for both proteins in producing brown dopamine-melanin (J. R. T., unpublished). The necessity of converting dopamine to NBAD prior to polymerization is surprising and its biological significance is unclear. Sequestration of dopamine as NBAD may be necessary to prevent its conversion to other molecules by alternative branches of the pathway (e.g. N-acetyl dopamine) (Brodbeck et al., 1998). Alternatively, because dopamine is a neurotransmitter, sequestration may be necessary to prevent toxic effects of dopamine accumulation.

#### A potential role for Tan in pigment patterning

*e* is necessary to specify the abdominal pigment stripe in *y* mutants. However, the uniform distribution of Ebony in the abdomen indicates that an additional protein downstream of *e* is also required. We propose that this factor is Tan, because *e* is epistatic to *t* and the biochemical function of Tan is dependent upon Ebony. This suggests that *t* promotes the abdominal pigment stripe in *y* mutants, and, in fact, in *y;t* mutants the stripe is reduced (J. R. T., unpublished). These observations suggest that the Tan protein may be expressed in a pattern similar to Yellow, a prediction that can be tested by cloning and characterizing the *t* gene.

#### Molecular mechanisms of pigment patterning

Taken together, our genetic experiments, prior biochemical studies, the expression patterns of Yellow and Ebony, and our predicted expression pattern of Tan suggest a molecular mechanism for generating the spatial distribution of pigment. Using the abdominal pigment stripe as an example, we propose that pigment patterning results from a combination of elevated levels of Yellow and Tan protein expression in cells that produce the pigment band, and low levels of Ebony protein expression throughout the segment (Fig. 7B). Assuming these expression patterns, we suggest that Yellow promotes the production of black dopa-melanin in the pigment stripe, cells expressing both Ebony and Tan (in the pigment stripe) produce brown dopamine-melanin, and cells expressing only Ebony (anterior to the pigment stripe) produce a tan colored NBAD polymer. The combination of these pigments would produce the final pigmentation of the adult fly (Fig. 7B).

#### Potential roles of *y* and *e* in intra- and inter-specific pigmentation variation

The functions of *y* and *e* in *D. melanogaster* suggest that these genes may be involved in intra- and inter-specific variation in pigmentation. Melanic pigmentation resembling the 'trident' pattern in *e* mutants is visible in some natural populations of *D.*

*melanogaster* and in closely related species (David et al., 1985; Capy et al., 1988). We found that in *D. melanogaster*, both Yellow and Ebony proteins are expressed in this pattern, suggesting that genetic variation in the expression of these genes may be responsible for phenotypic variation. Additionally, in *D. biarmipes*, Yellow and Ebony are expressed in patterns that correlate with an evolutionarily novel melanin pattern. This suggests that the molecular mechanisms responsible for pigment patterning in *D. melanogaster* are conserved in other species, and that changes in the distribution of Yellow and Ebony proteins may be involved in melanin pattern evolution.

#### Evolutionary change of spatial pigment patterns

Identification of the genetic changes responsible for pigmentation divergence may reveal general mechanisms underlying phenotypic evolution. Recently, Kopp et al. (Kopp et al., 2000) demonstrated that evolutionary divergence of sexually dimorphic abdominal pigmentation correlated with changes in the expression of *bric-a-brac* (*bab*), a gene that encodes a putative transcription factor that represses pigmentation and controls sex-specific abdominal morphology. This suggests that genetic changes altering the expression of a transcriptional regulator that controls multiple effector genes may contribute to phenotypic evolution. Ultimately, the effects of *bab* on pigmentation are likely to be mediated by changes in the expression of downstream structural genes (e.g. *y* and *e*) that are necessary for pigment production. It is also possible that genetic changes within the structural genes themselves may lead to altered expression patterns or functions responsible for phenotypic divergence. A crucial, unanswered question in evolutionary biology is whether genetic changes involved in phenotypic evolution occur more commonly at the level of pleiotropic regulators or of structural genes. The rapid evolution of pigment patterns within *Drosophila*, combined with a growing knowledge of the developmental and molecular mechanisms underlying pigment patterning, provide models with which to study this and other general questions regarding the genetic and molecular basis of phenotypic evolution.

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