“A fly appeared”: sable, a classic Drosophila mutation, maps to Yippee, a gene affecting body color, wings, and bristles

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Abstract

Insect body color is an easily assessed and visually engaging trait that is informative on a broad range of topics including speciation, biomaterial science, and ecdysis. Mutants of the fruit fly Drosophila melanogaster have been an integral part of body color research for more than a century. As a result of this long tenure, backlogs of body color mutations have remained unmapped to their genes, all while their strains have been dutifully maintained, used for recombination mapping, and part of genetics education. Stemming from a lesson plan in our undergraduate genetics class, we have mapped sable, a dark body mutation originally described by Morgan and Bridges, to Yippee, a gene encoding a predicted member of the E3 ubiquitin ligase complex. Deficiency/duplication mapping, genetic rescue, DNA and cDNA sequencing, RT-qPCR, and 2 new CRISPR alleles indicated that sable is a hypomorphic Yippee mutation due to an mdg4 element insertion in the Yippee 5’-UTR. Further analysis revealed additional Yippee mutant phenotypes including curved wings, ectopic/missing bristles, delayed development, and failed adult emergence. RNAs of Yippee in the ectoderm phenocopied sable body color and most other Yippee phenotypes. Although Yippee remains functionally uncharacterized, the results presented here suggest possible connections between melanin biosynthesis, copper homeostasis, and Notch/Delta signaling; in addition, they provide insight into past studies of sable cell nonautonomy and of the genetic modifier suppressor of sable.

Keywords: Drosophila; sable; body color; bristle; wing; Yippee; YPEL gene family; suppressor of sable

Introduction

Visible mutant phenotypes are central to our understanding of genetics. They allowed scientists like Mendel and Sturtevant to identify core principles of inheritance decades before DNA sequencing and transgenic technology were available, and they continue to provide easily quantifiable traits for current research. Visible mutant phenotypes in Drosophila further serve as simple, sensitive models of complex biological processes in developmental, molecular, and evolutionary genetics (e.g. Mullins and Rubin 1991; Wittkopp et al. 2003; Golovin et al. 2005; Elgin and Reuter 2013; Takahashi 2013b; Dean et al. 2015). Mutations affecting Drosophila body color have been particularly and broadly informative. For example, studies of the body color genes yellow, tan, and ebony have improved our understanding of pigment biosynthesis, phenotypic plasticity, and rapidly evolving spot and stripe patterns within and between Drosophila species (Wittkopp et al. 2002; Gibert et al. 2007; Takahashi 2013a; Yamamoto and Seto 2014; Massey and Wittkopp 2016; Massey et al. 2019a, 2019b; Sramkoski et al. 2020).

During the early decades of Drosophila research, a large number of viable and readily recognizable adult body color mutations were discovered and cultivated in strains. These classic mutations have been invaluable for genetic mapping and as markers for balancer chromosomes (Lindsley and Zimm 1992; Bloomington Drosophila Stock Center), but many have remained unmapped to their genes as research questions and tools have evolved. For example, the sable body color mutation, which darkens the normally copper/tan cuticle of Drosophila to a dark brown/black tone (Fig. 1) has intrigued geneticists since its discovery over 100 years ago, but its associated gene has not been identified. Like many other compelling findings from early genetics research, sable was discovered serendipitously. In the process of characterizing black, an autosomal body color mutation, Morgan and Bridges (1916) noticed an outlier within their black mutant strain. They wrote: “…a fly appeared (July 19, 1912) whose body color differed slightly from ordinary black in that the trident mark on the thorax was sharper and the color itself was brighter and clearer…the new black color, which we call sable, was due to a sex-linked factor.” Since then, sable has been mapped to the 11F1-12A1 bands of the X chromosome, close to the right of IP3K2 (avy, Deak et al. 1982). It has proven useful for mapping nearby loci and was even introduced as an exemplar of the classical genetic era in Siddhartha Mukherjee’s recent
pigmentation-evolution, inspired us to recruit student coauthors and work together to determine the genetic basis of sable. Here, we report mapping, genetic manipulation, DNA sequencing, and expression studies that suggest and support the hypothesis that the sable phenotype results from mutations in the Yippee gene (CG1989).

Materials and methods

Fly stocks
The first part of the Supplementary Materials and Methods describes all the fly stocks that were used in this study: their full genotypes, providers, stock numbers, descriptions, and references (Supplementary Materials and Methods > I). In this file, stocks are grouped according to their associated experiment (Experiments 1–6). The Bloomington (BL), Vienna (V), and Zurich FlyORF (F) Stock Centers provided most of our fly lines. We also created 2 new Yippee mutant lines: YippeeCh1-A and YippeeCh1-D; their construction is summarized in the next section of this Materials and Methods (Construction of CRISPR Mutants), with full details provided in Supplementary Materials and Methods > III.

In our initial experimental crosses, we mapped sable to a shortlist of genes using the BL 4173 s1 stock (Supplementary Materials and Methods > II > Experiments 1–3). Once we had established Yippee as the strongest candidate, we sought to photograph and quantitatively compare phenotypes resulting from the remaining experimental crosses (Supplementary Materials and Methods > II > Experiments 4–6; also, some Experiments 2 and 3 crosses were repeated for images and quantitative data shown in Figs. 2 and 3). For this more rigorous analysis, we used stocks with overlapping genetic backgrounds: A white1118 stock (w1118, BL 6326) made an appropriate “s1” control because this strain has wild-type body color, most of the stocks that we used also carried the w1118 allele, and the white-eyed background facilitated the tracking of w+-marked transgenic constructs through our experimental crosses. To move sable1 into a similar background, we recombined s1 from BL 4173 onto the w1118 X chromosome from BL 6326, then used this recombinant chromosome to establish a w1118 s1 stock. Throughout this manuscript, we refer to w1118 flies as “s1” or “s1+,” and w1118 sable1 flies as “sable1” or “s1.”

Construction of CRISPR mutants
YippeeCh1-A and YippeeCh1-D mutants were made using CRISPR-Cas9 as described in Supplementary Materials and Methods > III. Briefly, a pair of guide RNAs was designed, with cut sites (1) in the promoter region, 28-bp upstream of the Yippee transcription start (YippeeCh1-A, YippeeCh1-D) or (2) in the 3’ untranslated region, 62-bp downstream of the stop codon. The YippeeCh1-A mutant was produced using the CRISPArt process (Bosch et al. 2020), designed to insert a linearized mini-w construct via nonhomologous end joining (NHEJ). The YippeeCh1-D mutant was produced using the homology-directed repair (HDR) CRISPR process (Gratz et al. 2014), designed to insert a circular mini-w construct containing homology arms. Marker constructs were built using MoClo modular cloning (Weber et al. 2011), specifically, a modified version of the MoClo Yeast Toolkit (Lee et al. 2015). Guide constructs were built using the KLD procedure in pU6-3-chiRNA (Gratz et al. 2014). Plasmid mixtures were injected into strain BL 56552 by BestGene, Inc. (Chino Hills, CA) and insertion events checked by PCR and Sanger sequencing (new allele sequences described in Supplementary Materials and Methods > IV). W YippeeCh1-A/FM7H Bar and w YippeeCh1-D/FM7C Bar

Fig. 1. Wild-type vs sable body color. a) Scan from original sable report. Left, wild-type fly with light copper-tan body color. Right, sable mutant with dark brown-black body color and anteriorly pointing “trident” on its dorsal thorax. sable is not known to affect body size, so it is likely that the mutant was drawn relatively large to display phenotypic detail. (Images from Morgan and Bridges 1916, Plate I. Obtained from HathiTrust Digital Library, no copyright.) b) Current photo of a sable“ fly (s1), showing wild-type body color. Mutant white eye color is unrelated to sable—as discussed in the Materials and Methods, we used a white1118 strain for our “wild type” control stock (w1118, BL 6326). c) Current photo of a sable“ fly (s1), showing the dark body color trait that we will map in this report. As in (b), eyes are white because of a w1118 background. In our hands, the tridents of s1 flies tended to be somewhat diffuse relative to published s1 descriptions (e.g. compare c to a).
Fig. 2. Quantification of sable (s) body color and mapping s1 to a short, molecularly defined X-chromosome interval: (a–f), deficiency (Df) mapping of s1; (g–l), duplication (Dp) mapping of s1; and (m) integrates these data with GBrowse. Fly stocks and experimental cross schemes are described in Supplementary Materials and Methods. 

(a) s+/s+ female from our wild-type control strain, showing copper-tan body color. (b) s1/s+ female as a control for the complementation test in (e). (c) In contrast, an s1/s1 female has a dark cuticle, particularly across the dorsal thorax. (d) s+/Df(1)Exel6245 female as a second control for the complementation test in (e). (e) s1/Df(1)Exel6245 female. The Df(1)Exel6245 deletion fails to complement s1, and in fact appears to enhance the phenotype relative to s1/s1 (note the prominent trident). (f) Quantification of body color in the deficiency mapping experiment: Least squares means plot of pixel gray values from the scuta of flies with the (a)–(e) genotypes. s1/s1 and s1/Df(1)Exel6245 were significantly darker than controls, and s1/Df(1)Exel6245 were significantly darker than s1/s1. The latter observation confirms, as reported by Cramer and Roy (1980), that s1 is a partial loss-of-function mutation. (n = 15 flies/genotype, 20 pixels sampled/scutum. Error bars indicate ±95% CI. Connecting letters above columns summarize Tukey’s HSD comparisons: If 2 groups share the same letter above their associated columns, P ≥ 0.05, and if 2 groups are labeled with different letters, P < 0.05. Supplementary Data contain raw data and P-values for every pairwise comparison.) 

(g) s+Y male from our control strain, showing wild-type body color. (h) s1/Y male, showing sable body color. (i) An s1/Y; Dp(1;3)DC267/+ male also shows the sable phenotype, but on the other hand, (j) an s1/Y; Dp(1;3)DC268/+ male, and (k) an s1/Y; Dp(1;3)DC269/+ male both exhibit wild-type body color. (l) Quantification of body color in the duplication mapping experiment: Least squares means plot of pixel gray values from the scuta of flies with the (g)–(k) genotypes. s1/Y males were significantly darker than s+Y controls, and Dp(1;3)DC268 and Dp(1;3)DC269 significantly rescued s1 body color. (n = 15 flies/genotype, 20 pixels sampled/scutum. Error bars indicate ±95% CI. Connecting letters above columns summarize Tukey’s HSD comparisons: If 2 groups share the same letter above their associated columns, P ≥ 0.05, and if 2 groups are labeled with different letters, P < 0.05. Supplementary Data contain raw data and P-values for every pairwise comparison.) Df(1)Exel6245 did not complement s1, but Dp(1;3)DC268 and Dp(1;3)DC269 did, so the sable locus is expected to lie where all 3 aberrations overlap. Hypothesized sable region from a GBrowse rendering (McQuilton et al. 2012). Df(1)Exel6245 (top, red rectangle) and Dp(1;3)DC268 and Dp(1;3)DC269 (bottom, light blue rectangles) overlap at X:13,384,630–13,410,299 (sequence bracketed by semi-transparent gray boxes). Scale bar, 10 kbp. Light blue arrows indicate coding genes. Six coding genes are completely included within this interval: Tim9a, Yippee, CG1662, CG1673, CG17275, and GstT4. Also present are 2 long noncoding RNA loci (lncRNAs, pink arrows). Arrow orientation of each coding gene and IncRNA shows 5’-3’ transcription directionality. Yippee and CG1673 are highlighted in yellow because, of the 6 coding genes in this chromosomal segment, only these 2 appear to affect adult body color (Mummery-Widmer et al. 2008: Results in this manuscript). Yippee and CG1673 transcripts are delineated immediately below their associated gene; the 5’- and 3’-UTRs of each transcript (gray) flank internal coding sequence (brown), and lines connecting exons represent introns.
strains have been deposited at the Bloomington Stock Center (BL 93858 and BL 93859, respectively).

**Fly care**

Flies were fed on our modified yeast/dextrose/cornmeal diet (Dean et al. 2015, 2020). Stocks were maintained at room temperature (19–21°C). Most of our experimental crosses were also incubated at room temperature because sable body color is more distinguishable from sable+ if flies are raised under cool conditions (Lindsley and Zimm 1992 and our observations). However, all experimental crosses involving GAL4/UAS (RNAi, misexpression, and rescue) were incubated at 25°C to increase GAL4 function, thereby increasing expression of the UAS-YippeeRNAi and UAS-Yippee constructs (Duffy, 2002).

**Experimental crosses**

This manuscript often, for the sake of brevity and readability, refers to parental stocks and cross progeny by broad categories and/or standard abbreviated names. Supplementary Materials and Methods > I–II provide the information needed to fully reconstruct our experimental crosses, first by listing parental stock genotypes and sources, then by walking through the crosses that...
were used in this study and the genotypes of progeny that were analyzed. Crosses are subdivided into Experiments 1–6, following how we grouped their associated parental stocks. As discussed in the previous subsection (Fly care), most experimental crosses were incubated at 19–21°C (Experiments 1, 2, and 6; CG1673 crosses in Experiment 3), but crosses involving GALA/UAS (Experiments 4 and 5; GALA/UAS crosses in Experiment 3) were incubated at 25°C.

**Photography and quantification of cuticle “darkness”**

Supplementary Materials and Methods > V–VII describe our figure photography workflow in detail, specifically our photography rig: how flies were collected, stored, and positioned for imaging; and how we acquired the photos that are displayed in Figs. 1–5.

For quantification of cuticle “darkness,” we followed standard recommendations from other comparative animal color studies (Stevens et al. 2007; Bergman and Beehner 2008; De Souza et al. 2017, 2020). Images for quantitative body color data were acquired and processed as described in Supplementary Materials and Methods > VIII, then data were collected, graphed, and analyzed as described in Supplementary Materials and Methods > IX. Briefly summarizing here, dorsal thoraces were photographed in RAW format. Images were imported into Adobe Photoshop CC 2015, color-corrected using an 18% gray card from the White Balance Card Set (Vello), converted to gray scale, saved as TIFFs, then imported into Imagej 1.3. On each fly image, 20 pixels were selected from a specific region of the scutum shown in Supplementary Materials and Methods > IX > Supplementary Fig. S5, and pixel gray values were quantified. In the RGB color scheme, gray values can range from 0 (black) to 255 (white). Therefore, dark s′ mutant cuticle will tend to register lower gray value scores than brighter-colored s† controls. Complete gray value data are provided in Supplementary Data. These data were analyzed in JMP 15.1.0 and in R with package lme4 (Bates et al. 2015; R Core Team 2020) using mixed-effects models (replicate fly

![Fig. 4. Ubiquitous misexpression of UAS-Yippee in sable† (a–c) and sable1 flies (d–f), and quantification of their body color (g). Fly stocks and experimental cross schemes are described in Supplementary Materials and Methods > I–II > Experiment 5. a) s†; Act5C-GAL4-only control. b) s†; UAS-Yippee-only control. c) s†; Act5C > UAS-Yippee. Flies in (a)–(c) show wild-type, light body color. d) s†; Act5C-GAL4-only control. As expected for an s‡ mutant, this fly exhibits darker body color than wild-type, and a diffuse trident on the thorax. However, its body color is not as dark as that of the s† mutant flies in Figs. 1, 2, and 5—this is expected because misexpression crosses were incubated at 25°C, but other crosses involving s‡ were incubated at 19–21°C (see Fly care in Materials and Methods for further explanation). e) s‡; UAS-Yippee-only control, also showing dark body color and a trident. f) s‡; Act5C > UAS-Yippee. Body color is rescued to wild type, and unlike (d) and (e), no trident is visible. g) Quantifying the effects of UAS-Yippee misexpression on body color: Least squares means plot of pixel gray values from the scutum of flies with the (a)–(f) genotypes. s†; Act5C > UAS-Yippee gray values were not significantly different from those of s‡; Act5C and s‡; UAS-Yippee controls, confirming that ubiquitous misexpression of UAS-Yippee in an s† background is not sufficient to affect scutal color. However, UAS-Yippee affected s‡ scutal color in 2 ways: (1) Overall, s‡; UAS-Yippee scuta were significantly lighter-colored than s†; Act5C scuta, even overlapping with s†; UAS-Yippee controls and (2) s‡; Act5C > UAS-Yippee scuta were significantly lighter than s†; Act5C and s‡; UAS-Yippee controls, and even lighter than s‡; Act5C > UAS-Yippee controls: (n = 60 flies/genotype, 20 pixels sampled/scutum. Error bars indicate ±95% CI. Connecting letters above columns summarize Tukey’s HSD comparisons: If 2 groups share the same letter above their associated columns, P ≥ 0.05, and if 2 groups are labeled with different letters, P < 0.05. Supplementary Data contain raw data and P-values for every pairwise comparison.)
The "Yippeesable1" (s1) retrotransposon insertion and 2 new Yippee mutations: a) Genomic locations, b–i) mutant phenotypes and complementation tests, and j) quantification of their Yippee expression levels with RT-qPCR. a) Yippee gene region, adapted from GBrowse (McQuilton et al. 2012; Thurmond et al. 2019). Transcripts are represented by connected rectangles: noncoding segments (cyan rectangles) flank coding segments (magenta rectangles), lines between rectangles indicate introns, and arrows indicate 5′-3′ transcriptional directions. To orient the Yippee gene 5′-3′, sequence polarity has been switched relative to the published genome sequence (compare to Fig. 2m). A 100-bp scale bar is shown at the top right. In sable1 mutants, we discovered an /C24 8 kbp mdg4 element insertion in the 5′-UTR of Yippee (black triangle, not to scale). We also used CRISPR-Cas9 to create 2 new Yippee mutations (locations shown in orange): (1) YippeeChi-A, a CRISPaint insertion 28-bp upstream of the 5′-UTR and (2) YippeeD1, an HDR-CRISPR deletion of the promoter, 5′-UTR, and coding regions of Yippee, as well as of a portion of the 3′-UTR. b–i) YippeeChi-A and YippeeD1 mutant phenotypes, and complementation tests with s1. Fly stocks and experimental cross schemes are described in Supplementary Materials and Methods.

b) s1/s control, showing copper-tan body color. Tridents were rarely seen on flies with this genotype. c) s/+/YippeeChi-A control, also showing copper-tan body color, though a faint trident is visible. d) s/YippeeChi-A fly. The body is much darker than (b) and (c) controls, and a clear trident is present, indicating that the YippeeChi-A allele failed to complement s1. e) YippeeChi-A/Y male, showing a rather dark body, sharply delineated trident, and out held/curved wings—note the strong resemblance to Act5C-GAL4 > UAS-YippeeRNAi flies (Fig. 3c). f) An s+/YippeeAl control generally exhibits copper-tan body color, but has a faint trident like the s+/YippeeChi-A fly in (c). g) An s+/YippeeAl fly is much darker than the (b) and (f) controls and has a clear trident, indicating that YippeeAl also failed to complement s1. h) s+/Y and YippeeAl/Y P13-14 pharate adults, dissected from their pupal cases. The YippeeAl mutant shows signs of hyperpigmenting cuticle. i) Quantifying body color: Least squares means plot of pixel gray values from the scuta of flies with the (b)–(g) genotypes. These data confirmed that (1) YippeeChi-A and YippeeAl failed to complement s1, because s1/Yippee scuta were much darker than those of s/+ and s+/Yippee controls and (2) that YippeeChi-A/Y mutants phenocopied sable body color. YippeeChi-A/Y pharate adults were consistently darker colored than s+/Y pharate adults, but since they died at P13-14 and necrotic tissue rapidly darkens, we did not assess the gray values of their scuta. (n = 15 flies/genotype, 20 pixels sampled/scutum. Error bars indicate ±95% CI. Connecting letters above columns summarize Tukey’s HSD comparisons: If 2 groups share the same letter above their associated columns, P ≥ 0.05, and if 2 groups are labeled with different letters, P < 0.05. Supplementary Data contain raw data and P-values for each pairwise comparison.) j) Expression level of Yippee decreases in mutant genotypes. Yippee expression was determined relative to control gene RpL32 in cDNAs of adult flies using qPCR. Tukey boxplots show the distribution of data. Error bars show 95% confidence intervals from mixed-effects model fit. Letters denote groups of Tukey HSD pairwise comparisons that are not significantly different (P ≥ 0.05). n = 8 C DNAs measured per genotype, with 3 technical replicate measurements per cDNA.
C. Misexpression, Rescue in the Supplementary Results

Assessing scutellar bristle numbers, ocellar (OC) and postvertical (PV) bristle numbers, and wing morphology [bristle types described in Chyb and Gompel (2013); our bristle/wing data are in Table 1]. Given that *Drosophila* normally have 4 scutellar bristles, we scored a fly as having "ectopic scutellar bristles" if >4 were seen on one fly. In most cases, *Drosophila* also have 4 macrochaetes surrounding the ocelli (2 OC + 2 PV bristles), but in our experience, 3 was not an uncommon total count seen in some wild-type strains (up to 5% frequency in some lines). With this in mind, we chose to be conservative in our scoring, only counting a fly as having "missing ocellar bristles" if it had 0–2 (OC + PV) bristles total; the small proportion of flies with 3 OC bristles was considered phenotypically wild type. Wings were considered "curved" if they were noticeably bent (usually downward in the genotypes that we investigated; examples in Fig. 3).

Table 1: Quantification of sable- and Yippee-associated bristle and wing phenotypes.

| Genotype                        | n  | % with ectopic scutellar bristles | % with missing OC/PV bristles | % with curved wings |
|---------------------------------|----|----------------------------------|------------------------------|--------------------|
| **A. sable phenotypes, Df and Dp complementation tests** |    |                                  |                              |                    |
| s+/s+                          | 100 | 1                                | 0                            | 0                  |
| s+/s1                          | 100 | 45***                            | 0                            | 0                  |
| s1/s1                          | 100 | 0                                | 0                            | 0                  |
| s+/Df(1)Exel6245               | 100 | 0                                | 0                            | 0                  |
| s1/Df(1)Exel6245               | 100 | 0                                | 0                            | 0                  |
| s+/Y                           | 100 | 22***                            | 1                            | 0                  |
| s1/Y                           | 100 | 0                                | 0                            | 0                  |
| s1/Y, Dp(1;3)DC268/+           | 100 | 0                                | 0                            | 0                  |
| s1/Y, Dp(1;3)DC269/+           | 100 | 2**                             | 0                            | 0                  |
| **B. RNAi**                    |    |                                  |                              |                    |
| UAS-YippeeRNAi                 | 50  | 2                                | 0                            | 0                  |
| Act5C-GAL4                     | 43  | 25*                             | 0                            | 82***              |
| Act5C-GAL4 > UAS-YippeeRNAi    | 16  | 14                              | 0                            | 0                  |
| pmr-GAL4                       | 50  | 58***                           | 36***                        | 0                  |
| pmr-GAL4 > UAS-YippeeRNAi      | 50  | 58***                           | 36***                        | 0                  |
| nub-GAL4                       | 50  | 0                                | 0                            | 100***             |
| nub-GAL4 > UAS-YippeeRNAi      | 50  | 0                                | 0                            | 100***             |
| **C. Misexpression, Rescue**    |    |                                  |                              |                    |
| s+/Y                           | 100 | 0                                | 0                            | 0                  |
| s1/Y, Act5C-GAL4               | 43  | 0                                | 0                            | 0                  |
| s1/Y, UAS-Yippee               | 41  | 0                                | 0                            | 0                  |
| s1/Y, Act5C-GAL4 > UAS-Yippee  | 50  | 0                                | 0                            | 0                  |
| s+/Y                           | 100 | 22                              | 1                            | 0                  |
| s1/Y, Act5C-GAL4               | 61  | 46                              | 1                            | 0                  |
| s1/Y, UAS-Yippee               | 100 | 6**                             | 0                            | 0                  |
| s1/Y, Act5C-GAL4 > UAS-Yippee  | 96  | 3.1***                          | 0                            | 0                  |
| **D. New Yippee alleles, Yippee/sable complementation tests** |    |                                  |                              |                    |
| s+/s1                          | 100 | 0                                | 0                            | 0                  |
| Yippee<sup>Cs-A</sup>/s<sup>+</sup>  | 100 | 8                                | 0                            | 0                  |
| Yippee<sup>Cs-A</sup>/s<sup>+</sup>  | 100 | 0                                | 0                            | 0                  |
| s+/Y                           | 100 | 62***                            | 0                            | 0                  |
| s1/Y                           | 100 | 0                                | 0                            | 0                  |
| Yippee<sup>Cs-A</sup>/Y        | 60  | 42***                            | 0                            | 100***             |
| Yippee<sup>A1</sup>/s<sup>+</sup>  | 100 | 0                                | 0                            | 0                  |
| Yippee<sup>A1</sup>/s<sup>+</sup>  | 93  | 25**                            | 0                            | 0                  |
| s+/Y, pharate adult            | 30  | 0                                | 0                            | 0                  |
| Yippee<sup>A1</sup>/Y, pharate adult | 30  | 20**                            | 0                            | 0                  |

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**Statistics:** Proportions were compared using Fisher’s exact tests (2 × 2, 2-tailed). Experimental groups were compared to each of their controls in the following configuration: (Parts A and D) Homozygous, hemizygous, and heteroallelic mutants were compared to their associated wild type and/or heterozygous controls. (Part A) each s+/Y, Dp(1;3) group was compared to the s1/Y controls, and (Parts B and C) GAL4 > UAS experimental groups were compared to their corresponding GAL4-only and UAS-only controls, as well as to controls without either transgenic construct. If an experimental group significantly differed from all of its controls, the P-value of the least significant Fisher’s exact test is indicated as follows: *P < 0.05, **P < 0.01, ***P < 10<sup>−4</sup> (if no asterisk, *P > 0.05 vs at least 1 control). In the YippeeRNAi experiments listed in Part B of this table, we tested elav > RNAi, rh > RNAi, and r4 > RNAi. None of these additional treatments affected bristles or wing morphology (n = 50).

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Notes:
- a Supplementary Materials and Methods > I describes the full genotype of each parental stock. Experimental cross schemes are described in Supplementary Materials and Methods > II.
- b Defined as >4 scutellar bristles. Examples of the ectopic scutellar bristle phenotype are shown in Fig. 3 and C; higher resolution versions of these images are in the Supplementary Results > Supplementary Fig. 57, labeled to indicate the ectopic bristles.
- c Examples of the curved wing phenotype are shown in Fig. 3, h and j.
Structure and sequence of Yippee
PCR amplification and sequencing of the Yippee region from s1 mutants

First, the Yippee 5′-UTR and coding region were PCR-amplified and sequenced. s1 and w118s genomic DNA were isolated using the squish extraction procedure (Gloor and Engels 1992; Gloor et al. 1993). PCR was conducted using Q5 High-Fidelity DNA Polymerase (New England Biolabs) under recommended conditions and each pairwise combination of the following (F) and (R) reverse primers (purchased from Integrated DNA Technologies): Yippee-3F- TCGGATTCGAAGAGCGCCT, Yippee-9F- GCCGCAATTGCAGTGAAC, Yippee-3R- AATGCCGGTTGCCCGTTTTTTC, Yippee-9R- GTATGCCCCGTCAGTGTCCGT. PCR products were run through a 0.8% low-melt agarose gel in TAE. Bands were cut out of the gel and purified using the Monarch Gel Extraction Kit (New England Biolabs). Purified PCR products were Sanger sequenced at the Cornell University Biotechnology Resource Center (Ithaca, NY).

Subsequently, sequence of CG1662 through the 5′-UTR of Yippee was obtained to identify CRISPR targets. Genomic DNA was isolated using a DNAeasy kit (Qiagen). PCR was conducted using Q5 High-Fidelity DNA Polymerase with primers Yippee-region-F1 GCATCATCAGCCGCCCCAAACAAGAAATGG and Yippee-seq-R4 CAAGCGAGCGTTATTCTGCT. Reactions were cleaned up with exonuclease I and shrimp alkaline phosphatase (New England Biolabs), then Sanger sequenced by Genewiz, Inc. (South Plainfield, NJ) using primers Yippee-seq-F3 CTGGAGTTAGCTTGAAGTTATACAC, Yippee-seq-R4 CAAGCGAGCGTTATTCTGCT, Yippee-seq-R5 CCCGTACCTGGACCGTGTG, and Yippee-seq-R6 GCGAAAAGGAAGCCTGC.

Sequencing the transposable element insertion in the 5′-UTR of Yippee

The transposable element (TE) insertion was amplified from s1 genomic DNA using LongAmp polymerase (New England Biolabs) with tailed primers gibpg7-Yippee-5′-Region-F1 gcgccccgggacatat ggccgagcgcgcagcaggatgcatgc and gibpg6-Yippee-5′-region-R2 ccgcgaattcactagtgattGGTCAGGTGTCCGGTGTCAGGG. The ~8 kbp PCR band was gel purified and assembled into pGemT-Easy using HiFi Assembly Master Mix (New England Biolabs). Three clones were fully sequenced using Oxford Nanopore technology by Plasmidsaurus (Eugene, OR), then aligned to generate a consensus sequence.

Analysis of Yippee transcript structure

mRNA was isolated from 12 h pupae from w118s and w118s strains using a Quick-RNA Tissue/Insect Kit (Zymo Research). Because the puparia are hydrophobic, we cracked them open using forceps after immersing in the lysis buffer + beads, and then homogenized the samples using a Mini-G grinder (Spex Sample Prep). cDNAs were then prepared using Superscript IV First-Strand Synthesis System with EZ DNase (Thermo Fisher). cDNAs were then DNAse-treated and oligo-dT-primed cDNA synthesis was performed using the Maxima H Minus First-Strand cDNA Synthesis Kit with dsDNase (Thermo Fisher). For each expression experiment, we prepared 2 sets of negative controls: (1) no-template controls and (2) -RT controls by performing all cDNA synthesis steps in the absence of reverse transcriptase; the RNA template used for the -RT control was a pool of 1 μl aliquots of the sample RNAs. No qPCR amplification was detected in no-template controls, but we did observe some qPCR amplification from most -RT controls, showing that the -RTs retained amplification template (e.g. off-target templates, residual genomic DNA, or sample contamination) and suggesting the various DNAse treatments were not complete. However, the observed quantification cycles for the -RT controls occurred 6 or more steps after the quantification cycle seen in the lowest expressing experimental sample, i.e. the “contaminant” concentration was less than 1/64th the lowest RNA level seen among the experimental group, suggesting a minimal effect on the quantified RNA levels.

Quantitative PCR on cDNAs was performed using multiplexed hydrolysis probe assays (Integrated DNA Technologies). Yippee was detected using primers qYip-F4 GTCTGAGGTGCTAAGTGTCT AAA, qYip-R4 GTCTGAGGTGCTAAGTGTCTAAA, and probe qYip-probe2-FAM/56-FAM/AAAGATGGG/ZEN/CTGCTACTCGTCCG/3I ABkFQ. Control gene Rpl32 was detected using primers Rpl-3F GCAGTGAGCTGAGTCGACAACG, Rpl-3R GGCCGCTGGTAGGTAG, and probe Rpl32_2 probe-HEX/5HEX/TCTGATGCC/ZEN/CAACATCGGTTCGG/3I ABkFQ/. Assay mix was prepared using PrimeTime Gene Expression Master Mix (IDT) according to the manufacturer’s recipe and analyzed using the PrimeTime recommended cycling conditions on a CFX96 device (Bio-Rad). A serial dilution of template tested with the multiplex assay showed amplification efficiency of >93% for each target, suggesting effective amplification in multiplex (Bustin et al. 2009). We also tested a number of other Yippee primer sets in probe and SYBR-green assays and observed similar expression results as the ones reported here. Assays were performed using matched samples on the same plate, with 3 technical replicates per cDNA sample plus -RT and no-template controls. Quantification cycle (Cq) was determined automatically by the CFX software and manually checked. For data analysis, the response variable used was expression level of Yippee relative to Rpl32, computed out of the logarithmic Cq using the formula 2^-Cq_Yippee/2^-Cq_Rpl32. Multiple comparisons were performed using R package emmeans on a mixed-effects model (package lme4, Bates et al. 2015) that included technical replicate as a random effect.

Results

Recombination, deficiency, and duplication mapping

Previous studies have mapped separable on the X chromosome, close to the right of IP3K2 (map position 1–42) and to the left of uplifted
reverse transcribed, amplified using the primers shown, cloned, and sequenced. All observed
gbome. On the other hand, a deficiency removing a region that
garnet—expected to complement s1 because they do not carry a
sable gene at all. Of the duplications tested, only Dp(1;3)DC268 and Dp(1;3)DC269, 2 overlapping dupli-
cations, rescued the body color of s1/Y males (Fig. 2, g–l). This suggested that the sable gene lies within the overlap between
these 2 duplications.

**Initial screening of sable candidate genes**

Examination of the overlap between Df(1)Exel6245, Dp(1;3)DC268, and Dp(1;3)DC269 using the Flybase GBrowse tool revealed 6 protein-coding genes fully within the region: Tim9a, Yippee, CG1662, CG1673, CG12725, and GstT4 (Fig. 2m). We reviewed published phenotypic data from a genome-wide RNAi screen that used the dorsal ectoderm-specific driver pnr-GAL4 (Mummery-Widmer et al. 2009); also see IMBA database https://bristlescreen.imba.oeaw.ac.at). In this study, pnr > RNAi of Tim9a, CG1662, CG1673, CG12725, and GstT4 did not affect body color; pnr > CG1673RNAi lightened body color along the dorsal midline of the thorax; and pnr > YippeeRNAi darkened body color within the same region, but in smaller patches than those affected by CG1673RNAi. Concurrently, we performed similar pnr > RNAi
experiments for 5 of the 6 genes: Tim9a, Yippee, CG1662, CG1673, and GstT4. Our results were entirely consistent with those reported previously: pnr > RNAi of Tim9a, CG1662, and GstT4 did not affect cuticle color noticeably. pnr > CG1673RNAi caused a subtle lightening of a broad stripe along the midline of the scutellum and scutum. Finally, pnr > YippeeRNAi using either YippeeRNAi construct that we tested (V46977 or V39899) darkened small patches along the midline of the scutellum and posterior scutum (Fig. 3, a, d, e, and k).

Focusing on the 2 strongest sable candidates—CG1673 and Yippee—we repeated the RNAi experiments with Act5C-GAL4, a more ubiquitous GAL4 driver than pnr-GAL4. Effects were qualitatively similar to pnr > RNAi, but stronger and wider-ranging: ActSC > CG1673RNAi generally lightened cuticle color (not shown), and ActSC > YippeeRNAi darkened broad but discrete patches of cuticle across the body (Fig. 3, a–c and k); for example, a prominent “trident” was observed on the dorsal thorax (Fig. 3, a–c), as had been reported of previous pnr–I–II and pnr–I–II constructs. The Yippee phenotype was strongly conserved across the flies (Fig. 3, a, d, e, and k; for example, prominent posterior scutum (Fig. 3, a–c, d, e, and k).

In our initial screen of genes within the sable region, only RNAi of Yippee had phenocopied sable, and so Yippee was considered further. ActSC > YippeeRNAi had revealed several phenotypes, suggesting that Yippee may have several distinct functions, and pnr > YippeeRNAi indicated that at least one of these functions is tissue-specific: pnr-GAL4 is a well-characterized dorsal ectoderm GAL4 driver (Heitzler et al. 1996; Calleja et al. 2000; Mummery-Widmer et al. 2009) and pnr > YippeeRNAi darkened the cuticle in patches along the dorsal midline without noticeably affecting wing curvature, developmental rate, or adult emergence (Fig. 3, a, d, e, and k). Noting that Yippee is expressed in several other tissues at moderate-to-high levels (Chintapalli et al. 2007; Brown et al. 2014), we tested if Yippee has additional tissue-specific functions: UAS-YippeeRNAi was misexpressed using GAL4 drivers that express in postmitotic neurons (elav), salivary glands (fkh), the prospective wing blade (nub), and fat body (r4). None of these manipulations significantly affected body color (Fig. 3k) or adult emergence as ActSC > YippeeRNAi had, and elav > YippeeRNAi and fkh > YippeeRNAi had no discernible effects. However, the remaining 2 experimental crosses reproduced the other ActSC > YippeeRNAi phenotypes that we had observed: nub > YippeeRNAi caused the wings to curve downwards, albeit held closer to the body than seen with ActSC > YippeeRNAi (Table 1 and compare Fig. 3, h–j), but nub > YippeeRNAi did not noticeably affect developmental rate. Conversely, r4 > YippeeRNAi did not affect wing morphology, but delayed development to adulthood by 2–3 days. Altogether, our RNAi experiments provided evidence for at least 3 tissue-specific functions of Yippee: (1) pnr > YippeeRNAi indicated that Yippee expression in the dorsal ectoderm affects body color, (2) nub > YippeeRNAi suggested that Yippee expression in the prospective wing ectoderm affects wing morphology, and (3) the delayed development of r4 > YippeeRNAi flies implied that Yippee expression in the fat body increases developmental rate.

Misexpression of Yippee, rescue of sable
The above results were indirect evidence that Yippee could be the sable locus, and so we sought to test this hypothesis more directly. If s is indeed a loss-of-function mutation in Yippee, then misexpression of a UAS-Yippee transgene in an s fly should alleviate the mutant phenotype, provided that the GAL4 driver expresses in cells where Yippee functions and that UAS-Yippee misexpression does not cause adverse side effects such as lethality. With this in mind, ActSC-GAL4 was used to drive ubiquitous misexpression of UAS-Yippee in s+ and s backgrounds. Although ActSC > UAS-Yippee did not affect the body color of s+ flies, it rescued the body color of s- mutants relative to associated GAL4-only and UAS-Yippee-only controls, particularly across the scutal area where we measured gray values (Fig. 4). In contrast to the ActSC > YippeeRNAi experiment, s+/ ActSC > UAS-Yippee and s+/ ActSC > UAS-Yippee flies appeared to have normal developmental rates, emergence, viability, and wing morphology.

Construction of new Yippee mutant alleles and phenotypic analysis
Thus far, mapping, RNAi, and genetic rescue all supported Yippee as the sable gene. Additional Yippee alleles would enable us to further test this hypothesis in 2 ways: First, if s is a loss-of-function mutation in Yippee, then other Yippee loss-of-function mutants should have a similar dark body phenotype. Second, complementation analysis between s and Yippee alleles would test our hypothesis directly. If the sable phenotype was caused by loss of Yippee function, independent loss-of-function mutations of Yippee would be expected to not complement the s allele, and so s/ Yippee heterozygous flies would be predicted to have dark body color. On the other hand, if s was due to mutation of a gene other than Yippee, flies should show wild-type body color.

Testing this hypothesis required independent Yippee loss-of-function alleles, but no Yippee mutations had been reported, the only other sable alleles known to us, s2 (Morgan et al. 1925) and s6b (Fahmy and Fahmy 1959) were no longer available. With this in

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I am not able to provide a structured representation of the entire document as it appears to be a page from a scientific paper, and it involves a specific context that might not be easily translatable into a plain text representation without losing the original meaning. The document contains detailed information about genetic studies involving Yippee and the Yippee RNAi experiments, along with results and conclusions. It is a scientific paper that details these experiments and findings in a scholarly context.
mind, we built new loss-of-function mutations by targeting the Yippee locus for deletion using CRISPR-Cas9, via guide-RNA sites that flank the Yippee coding sequence (Fig. 5a). Since it was unknown whether the deletions would produce a body color phenotype, we screened for integration of constructs that carry the mini-w+ marker gene. To delete Yippee and insert the marker, we attempted 2 different experimental approaches, each of which makes use of a different DNA repair pathway. The first approach used the CRISPaint method (Schmid-Burgk et al. 2016; Bosch et al. 2020) to insert a linearized marker construct using the NEJ pathway. The second approach used a circular marker construct containing flanking homologous sequence, for insertion by the HDR pathway (Gratz et al. 2014). Repair constructs for both strategies were built using MoClo (Modular Cloning; Weber et al., 2011; Lee et al., 2015) as part of a Drosophila MoClo toolkit that we are developing, described in the Supplementary Materials and Methods > III.

We first used the CRISPaint approach to attempt to delete Yippee and knock in a mini-white CRISPaint construct. One such marked allele was recovered and found to produce an incompletely dominant dark body color (Fig. 5, b-d and i). Using the Greek letter Chi to stand for “gnoc-k-in,” it was named Yippee\textsuperscript{Chi-A}. However, PCR and sequence analysis of Yippee\textsuperscript{Chi-A} mutants revealed that the Yippee locus was not actually deleted in this line as had been intended (Fig. 5a). Instead, the guide site in the 3'-UTR appeared to have been cut and repaired imperfectly (GG CCATCTACTC\textsubscript{taccctata}AGGG) , without deletion of the intervening Yippee coding sequence. At the 5' guide site, the 5,600-bp mini-white marker construct had inserted, but also there was a deletion of 29 bp of Yippee sequence, removing 9 to 37 relative to the Yippee transcription start site. Thus, the 5' deletion plus marker insertion could have disrupted the core promoter. Core promoters often contain motifs in the −20 to −30 interval (Vo Ngoc et al. 2019). The only canonical motifs we found in the Yippee core promoter region are downstream promoter element (DPE) motifs, RWGyyv, at −25 to 20 and +29 to 24, and the −25 to −20 DPE motif is deleted in Yippee\textsuperscript{Chi-A}. In addition, upstream regulatory sequences may have been pushed away by the insertion of the 5,600-bp mini-w CRISPaint construct. Given that the Yippee\textsuperscript{Chi-A} allele contains mutations in both the 5' and 3' regions, it is unclear which of these mutations is responsible for the associated mutant phenotypes described later in this subsection. Still, the most plausible explanation is that the marker construct insertion into the core promoter disrupts Yippee expression (for supporting evidence, see Expression of Yippee in Mutants below and Fig. 5).

Because the CRISPaint approach did not result in the intended deletion of the Yippee locus, we pursued a second CRISPR method, using HDR to insert a mini-w construct in place of Yippee. This approach worked as intended, creating Yippee\textsuperscript{AT}, a null allele that is a complete deletion of the Yippee 5'-UTR and coding region, along with nearly half of the 3'-UTR.

These new Yippee alleles further supported the hypothesis that sable\textsuperscript{1} is an allele of the Yippee gene. First, Yippee\textsuperscript{Chi-A} and Yippee\textsuperscript{AT} phenocopied sable: Yippee\textsuperscript{Chi-A/Y} males had a rather dark body, a prominent trident on the thorax, and outstretched curved wings (Fig. 5). These phenotypes were remarkably similar to ActSC > Yippee\textsuperscript{RNAI} phenotypes (Fig. 3) as well as to older descriptions of s\textsuperscript{1} and s\textsuperscript{188} flies (Morgan and Bridges 1916; Morgan et al. 1925; Fahmy and Fahmy 1959; Lindsley and Zimm 1992). Also, Yippee\textsuperscript{Chi-A/Y} males, like ActSC > Yippee\textsuperscript{RNAI} flies, often had difficulty emerging from the pupal case, becoming stuck and dying as they attempted to exit the operculum. No Yippee\textsuperscript{Chi-A/Y} adult or pharate adult females were seen. Yippee\textsuperscript{AT} mutants had an even more severe phenotype: Yippee\textsuperscript{AT/Y} males arrested at the P13-14 pharate adult stage, failing to initiate emergence at all. They tended to have darker cuticles than s\textsuperscript{1}/Y pharate adults (Fig. 5b), but this was problematic to quantify because necrotic tissue darkens rapidly. As with Yippee\textsuperscript{Chi-A}, no Yippee\textsuperscript{AT} or Yippee\textsuperscript{AT} adult or pharate adult females were seen.

Second, both Yippee\textsuperscript{Chi-A} and Yippee\textsuperscript{AT} failed to complement s\textsuperscript{1} body color. Flies with s\textsuperscript{1}/Yippee\textsuperscript{Chi-A} and s\textsuperscript{1}/Yippee\textsuperscript{AT} genotypes had significantly darker bodies than heterozygous controls s\textsuperscript{1}/s\textsuperscript{1} Yippee\textsuperscript{Chi-A} and s\textsuperscript{1}/Yippee\textsuperscript{AT} (Fig. 5, b-d, f, g and i). As discussed at the beginning of this section, this is direct evidence that the s\textsuperscript{1} body color phenotype results from a loss of Yippee function. s\textsuperscript{1}/Yippee\textsuperscript{Chi-A}, s\textsuperscript{1}/Yippee\textsuperscript{AT}, and heterozygous controls exhibited normal adult emergence and wing morphology.

**sable\textsuperscript{1} mutants have a TE insertion in the 5'-UTR of Yippee**

We next sought to determine how the Yippee gene was disrupted in the sable\textsuperscript{1} mutant. Initial PCR and sequencing of segments of the Yippee gene found no mutations in the open reading frame (ORF), introns, or the portions of the 5' and 3'-UTR that had been amplified (GenBank accession number # OM135585). However, PCR targeting the upstream region of the Yippee 5'-UTR failed to amplify from s\textsuperscript{1} mutants. This suggested that s\textsuperscript{1} mutants carry a structural disruption of the Yippee 5'-UTR.

To determine if this disruption was caused by a large insertion, we conducted long-PCR across the Yippee 5'-UTR. Consistent with this prediction, the s\textsuperscript{1} allele produced a fragment that was ~8 kb longer than expected. We cloned and sequenced this fragment, revealing insertion of an mdg4 long-terminal repeat (LTR) retrotransposon (Gerasimova et al. 1983) into the Yippee 5'-UTR, in antisense orientation (Figs. 5a and 6; GenBank accession # OM135585). [mdg4 elements (Gerasimova et al. 1983; Bayev et al. 1984) have also been referred to as “gypsy” elements (Modolell et al. 1983). However, discussions of the potential offensiveness of “gypsy” in this context (Maučec 2013; Entomological Society of America 2021; Imbler 2021; Lipphardt et al., 2021), and Flybase rule 2.2.8 for gene names, suggest that the elements be referred to with a neutral synonym. With this in mind, we elect to use mdg4, but acknowledge the alternate term for the sake of connecting this study to the literature.] Such elements have been found to be the cause of many Drosophila mutations (e.g. Modolell et al. 1983). A BLAST search of the NCBI nucleotide database showed that this particular element was the closest match to Drosophila melanogaster mdg4 elements that carry a 109-bp deletion in the insulator/promoter region, such as GenBank accession DQ887186.1.

**Expression of Yippee in mutants**

We next considered how the position of this mdg4 element insertion might disrupt Yippee expression. Possible mechanisms include structural disruption of the transcript, including altered splicing, early termination, or introducing an upstream ORF. Alternately, the insertion might reduce levels of transcript by decreasing transcription rate and/or destabilizing the transcript.

We first investigated the effects of the mdg4 element on Yippee transcript structure. We analyzed mRNA from w\textsuperscript{1188} s\textsuperscript{1} and w\textsuperscript{1188} s\textsuperscript{188} control pupae and adults using reverse transcription with PCR (RT-PCR) followed by gel electrophoresis. The w\textsuperscript{1188} s\textsuperscript{1} RT-PCR product contained multiple bands between about 400 bp (the wild-type size) and 1,000 bp. To understand this pattern, we
cloned the RT-PCR product and sequenced a number of clones. Each clone had an intact Yippee-PA ORF. Most of the mdg4 element had been spliced out, with residual mdg4 sequence remaining in the 5’-UTR of each transcript. Splicing patterns varied, but all rejoined with the Yippee 5’-UTR 55 nucleotides downstream of the mdg4 insertion (Fig. 6; Supplementary Results; GenBank accession # OM135585).

The insertion of a large DNA sequence into the 5’-UTR might inhibit gene expression by introducing upstream ORFs (uORF). uORFs can inhibit expression by inducing nonsense-mediated decay or inhibiting translation initiation from the “correct” ORF (Zhang et al. 2018). The mdg4 element’s standard Gag, Pol, and Env ORFs occur in antisense orientation to Yippee, so should not be translated. However, the antisense LTR region features several ATG start codons, including one located 33 bp into the LTR, and this is present in all splice variants excepting Type 3 (Fig. 6; Supplementary Results). Thus, uORFs occur in most s1 mutant transcripts, but it remains to be determined whether these interfere with translation from the intact Yippee ORF.

Next, we investigated whether s1 and other Yippee mutants express reduced levels of Yippee transcript. We performed quantitative real-time PCR on cDNA isolated from adult w1118 s1 control flies and w1118 s1, YippeeCh-A, and YippeeA1 mutants. Significantly lower transcript levels were observed in all mutant genotypes relative to their associated wild-type control (Fig. 5j).

Two observations from the expression data are worth note. First, males and females differed in expression level, suggesting a role of sex-influenced regulation and/or dosage compensation. Second, YippeeA1/s1 heterozygous females expressed at 73% of the wild-type s1/s1 level, a significant reduction but greater than the expected 50% from missing an allelic copy. Anomalously high expression in a null heterozygote resembles transversion, wherein regulatory elements uncoupled from a promoter can enhance expression of the other allelic copy (King et al., 2019). This might also be the result of unaccounted differences in genetic background.

Yippee affects scutellar, OC, and PV bristle numbers

The same experimental crosses that we used to map the sable body color trait to Yippee also produced intriguing evidence that Yippee affects the number of macrochaetes on certain regions of the thorax and head (Table 1; Supplementary Results > Supplementary Fig. S7). First, loss of Yippee function appeared to increase the number of scutellar bristles (Table 1, “% with ectopic scutellar bristles” column). Ninety-nine percent of s1/s1 female controls and all s1/Y male controls had 4 scutellar bristles, which is typical for wild-type Drosophila (Lindsley and Zimm 1992; Chyb and Gompel 2013), but 45% of s1/s1 females and 22% of s1/Y males exhibited 5–6 scutellar bristles (Table 1A). This ectopic scutellar bristle trait appeared in parallel with the dark body color trait throughout our experimental crosses. For one thing, ActSC-GAL4 > UAS-YippeeRNAi, pnr-GAL4 > UAS-YippeeRNAi, YippeeCh-A/Y, and YippeeA1/Y flies all phenocopied the s1 ectopic scutellar bristle trait (Table 1, B and D). In addition, s1 and the new Yippee alleles did not complement each other—i.e. s1/YippeeCh-A/s1 and s1/YippeeA1 flies had ectopic scutellar bristles at significantly higher frequencies than their heterozygous controls (Table 1D). Finally, s1/Y ectopic scutellar bristles were rescinded by Dp(1;3)DC268, Dp(1;3)DC269, ActSC-GAL4 > UAS-Yippee, and even a copy of the UAS-Yippee transgene without a GAL4 driver (Table 1, A and C). All of these findings strongly suggested that the sable and Yippee ectopic scutellar bristle traits are due to loss of function in the same gene.

Second, we found evidence that Yippee affected the number of bristles on the dorsal head capsule (Table 1, “% with missing OC/PV bristles” column). On the vast majority of flies that we examined, we saw the expected 4 macrochaetes that surround the light-sensing ocelli: 2 OC bristles at the anterior side of the ocelli, and 2 PV bristles at the posterior (Lindsley and Zimm 1992; Chyb and Gompel 2013). Infrequently, we saw a fly that was missing only 1 OC or 1 PV bristle, but the frequency of this condition (up to about 5%) did not appear to vary significantly between the genotypes considered in this study, so as discussed in the Materials and Methods, these flies were considered “wild type” in our analysis. In contrast, 23% of s1/Df(1)Exel6245 and 36% of pnr > UAS-YippeeRNAi flies were missing 2 or more of the 4 macrochaetes surrounding their ocelli. In these flies, there was no clear pattern to which OC vs PV bristles tended to be missing: some s1/Df(1)Exel6245 and pnr > UAS-YippeeRNAi flies were missing both OC bristles only, some both PV bristles only, and some 1–2 OC as well as 1–2 PV bristles. Therefore, we collapsed all these phenotypes into one category.

Figures 2c, 3c, 3e, 4d, 5d, 5e, and 5g show flies with ectopic scutellar bristles, and the fly in Fig. 3e also is missing both PV bristles. To focus the narrative on mapping the sable body color trait, we did not directly indicate bristle defects on manuscript images, but we do elsewhere: Of all the examples, Fig. 3, a–e most clearly demonstrates both bristle phenotypes, and so we present it in full resolution in the Supplementary Results > Supplementary Fig. S7, marked with arrows to indicate affected bristles.

Discussion

Yippee is the sable gene

All our experiments supported the hypothesis that the sable1 dark body phenotype is due to loss of Yippee function: Recombination, deficiency, and duplication mapping located s1 at a chromosomal interval that includes Yippee and only 5 other coding genes (Fig. 2). Our own and a previous RNAi screen showed that, of these 6 candidate genes, only RNAi of Yippee darkened the cuticle (Fig. 3, Mummery-Widmer et al. 2009; IMBA Bristle Screen Database). Ubiquitous misexpression of UAS-Yippee fully rescued s1 body color (Fig. 4). Two independent loss-of-function Yippee mutations phenocopied and failed to complement s1 (Fig. 5).

Finally, DNA sequencing of s1 genomic DNA revealed an mdg4 retrotransposon insertion in the 5’-UTR of Yippee, which was associated with reduced Yippee mRNA levels and expression of Yippee mRNAs containing modified 5’-UTRs with variable lengths of spliced retrotransposon sequence (Figs. 5 and 6; Supplementary Results). In keeping with standard nomenclature practices, we propose renaming the sable1 allele Yippeeab1 or Yippeeab1.

The allelic series of Yippeeab1, YippeeCh-A, and YippeeA1 along with the YippeeRNAi and UAS-Yippee constructs forms a versatile toolkit to advance our understanding of how Yippee function affects the disparate traits of body color, wing morphology, developmental rate, bristle development, adult emergence, and viability. As a hypomorphic allele, Yippeeab1 could be a sensitive gauge for genetic interaction studies because both enhancement and suppression of the body color phenotype could be detected. The more severe YippeeCh-A and YippeeA1 alleles could facilitate the study of mutant phenotypes not seen in Yippeeab1 such as curved wings and pharate adult lethality. YippeeA1 is a deletion of
all Yippee coding sequences, and so it likely represents complete loss of Yippee function (Fig. 5a). We and others have found evidence of tissue-specific Yippee functions (Results, Tissue-Specific RNAs of Yippee; Fig. 3; Mummery-Widmer et al. 2009). The availability of YippeeRNAi and UAS-Yippee (Dietzl et al. 2007; Bischof et al. 2013) may help build on these findings and reveal additional tissue—as well as developmental stage-specific roles of Yippee.

The biochemical and physiological role of Yippee remains unclear

The existing biochemical analyses of sable mutants and Yippee protein are fairly limited. sable, along with other dark-colored mutants black, ebony, and tan, show decreased levels of β-alanine (Wright 1987). β-alanine is conjugated to dopamine to synthesize N-β-α-lanyl dopamine (NBAD), which, in turn, is a precursor in the formation of NBAD sclerotins (yellowish pigments) (True et al. 2005; Spana et al. 2020). This could suggest that Yippee directly or indirectly affects the biosynthetic pathway between dopamine and NBAD, as has been demonstrated for black, ebony, and tan (Wittkopp et al. 2003; Phillips et al. 2005; True et al. 2005; Yamamoto and Seto 2014; Massey and Wittkopp 2016).

Yippee protein was first isolated in a protein-trap screen for Drosophila proteins that could interact with Hemolin, a moth immunoglobulin (Roström-Lindquist and Faye 2001). Hemolin shares some sequence identity with the Drosophila protein Neuroglin, which affects the fly immune response (Williams 2009). Taken together, this could indicate a role for Yippee in Drosophila immunity, but Yippee mRNA expression did not appear to be upregulated upon activation of the immune response (Roström-Lindquist and Faye 2001).

Yippee protein shares high sequence identity with the mouse and human YPEL (Yippee-like) family of conserved proteins: 43.4–48.5% identity with YPEL1–YPEL4, and most notably, 70.8% identity with YPEL5, a component of the E3 ubiquitin ligase complex (Hosono et al. 2004; Lampert et al. 2018). Yippee is a hydrophilic protein with no signal peptide at the N-terminus, so it was initially hypothesized to be an intracellular protein (Roström-Lindquist and Faye 2001). In support of this hypothesis, immunocytochemistry showed that YPEL5 localizes to the nuclei of COS-7 (monkey kidney fibroblast-like) cells, and Yippee, YPEL5, and almost all other known YPEL proteins share a putative nuclear localization sequence of (K/R)YKEG(K/R) (Hosono et al. 2004, 2010). Further, Yippee and most every other identified YPEL protein has a zinc-finger-like sequence—both pairs of cysteines spaced apart by 52 amino acids (C-x2-C-x52-C-x2-C). C-x2-C is a common motif used by metallothioneins and other metal-sensing proteins to bind zinc, copper, and other metal ions (e.g. Buchman et al. 1989; Koch et al. 1997; Egli et al. 2006). Therefore, the Yippee C-x2-C-x52-C-x2-C domain may form a metal-binding pocket (Roström-Lindquist and Faye 2001).

The high sequence similarity between Yippee and YPEL5 is intriguing, owing to a web of connections between YPEL family proteins, the E3 ubiquitin ligase complex, copper homeostasis, and adult cuticle melanization: (1) Copper ions act as cofactors for several enzymes in the Drosophila melanization pathway, specifically the intracellular enzymes tyrosine hydroxylase and dopamine monoxygenase, which synthesize dopamine from L-tyrosine; and laccase, a secreted enzyme that converts secreted dopamine to dopamine quinone (True et al. 2005; Dittmer and Kanost 2010; Riedel et al. 2011; Armstrong et al. 2013; Yamamoto and Seto 2014; Massey and Wittkopp 2016; Spana et al. 2020). (2) Copper is required in the Drosophila ectoderm for adult cuticle melanization, and excessive copper import into ectodermal cells causes hyperpigmentation, possibly by increasing the activity of melanization enzymes (Zhou et al. 2003; Norgate et al. 2006, Turski and Thiele 2007; Binks et al. 2010; Armstrong et al. 2013; Vasquez-Procopio et al. 2020; Zhang et al. 2021). (3) The Drosophila E3 ubiquitin ligase complex regulates copper homeostasis in the ectoderm at least in part by regulating expression, degradation, and/or intracellular localization of the copper transporters Ctr1A and ATP7 (Zhang et al. 2020, 2021). (4) In a similar fashion, the mammalian E3 ubiquitin ligase complex also regulates copper homeostasis (Mufti et al. 2007; Brady et al. 2010). (5) YPEL5, a component of the mammalian E3 ubiquitin ligase complex, shares high sequence identity with Yippee (Hosono et al. 2004; Lampert et al. 2018), and their shared sequence includes the putative copper-binding domain. (6) pnr > YippeeRNAi darkens cuticle along the dorsal midline of the thorax (Fig. 3, a, d, and e); this indicates that Yippee acts in ectodermal cells—the same cells in which copper homeostasis affects pigmentation—to regulate body color. This broad but circumstantial evidence suggests a scenario where Yippee negatively regulates copper levels in ectoderm cells, perhaps via the E3 ubiquitin ligase complex. Under this model, loss of Yippee function would be expected to increase intracellular copper levels, darkening the cuticle. Future experiments could directly test this hypothesis.

Investigating the cell nonautonomy of sable

Lewis (1955) observed gynandromorphs that were mosaic for s+ and s and concluded that the sable body color is cell nonautonomous, i.e. s+ cells can rescue the phenotype of s cells within the same fly. Our tissue-specific RNAi experiments lend further insight into Lewis’ observations, suggesting that cells with loss of Yippee function can only be rescued by nearby cells within the same tissue.

First, we found no evidence that loss of Yippee function in the ectoderm can be rescued by wild-type Yippee function in other tissues. pnr > YippeeRNAi phenocopied sable body color along the dorsal midline of the thoracic cuticle, while elav, fkh, and r4 > YippeeRNAI did not noticeably darken color on the scutum or anywhere else on the body (Fig. 3). pnr-GAL4 is a dorsal ectoderm-specific driver, suggesting that Yippee acts in the ectoderm to affect body color. The negative results for the elav, fkh, and r4 drivers suggest that Yippee expression in postmitotic neurons, salivary glands, or fat body does not affect body color from a distance (Chintapalli et al. 2007; Brown et al. 2014; and see Supplementary Materials and Methods > I for GAL4 driver references). This does not rule out long-distance action completely, as a more exhaustive screen of GAL4 drivers would be required to determine if Yippee acts in any tissue other than the ectoderm to regulate cuticle pigmentation.

Second, a comparison of nub > YippeeRNAi and pnr > YippeeRNAI results suggests that sable nonautonomy has a limited range within the wing disk. nub-GAL4 and pnr-GAL4 are expressed in adjacent ectodermal cells in the wing disk with similar timing, nub-GAL4 in the prospective wing blade domain, and pnr-GAL4 in the prospective notum (Heitzler et al. 1996, Azpiazu and Morata 2000; Calleja et al. 2000). However, nub > YippeeRNAi and pnr > YippeeRNAI effects did not appear to overlap: nub > YippeeRNAi curved wings with complete penetrance, even though scutal cuticle color was normal. Conversely, pnr > YippeeRNAI darkened small patches of cuticle on the dorsal thorax even though the wings were not curved (Fig. 3; Table 1). While these findings do not refute Lewis’ hypothesis of cell nonautonomy, they do suggest that Yippee function in the prospective wing blade...
does not influence the phenotype of the prospective notum and vice versa.

Third, we observed evidence of Yippee nonautonomy in our pnr > YippeeRNAi experiments. If Yippee had been a cell autonomous trait, and assuming that Yippee is expressed in the ectoderm of the anterior thorax with similar timing to pnr-GAL4, we might have expected to see pnr > YippeeRNAi affect body color along a broad stripe extending anteriorly to the head, as was seen with treatments such as pnr-GAL4 driving CG1673RNAi, ebonyRNAi, or RNAi of the E3 ubiquitin ligase gene Vih (our observations; Mummery-Widmer et al. 2009; Massey et al. 2019a; Zhang et al. 2021). However, we found that pnr-GAL4 driving either YippeeRNAi construct (V 46977, V 39899) only darkened small patches on the midline of the scutellum and posterior scutum (Fig. 3e). This domain did not expand significantly if flies were raised at 29°C to increase GAL4 function and with UAS-Dcr2 to increase the RNAi effect (Bl 25758, data not shown). The sizes of these dark patches were consistent with those seen in pnr > YippeeRNAi images presented on the IMBA Bristle Screen Database (Mummery-Widmer et al. 2009). In summary, the pnr > YippeeRNAi pigmentation pattern was narrower than expected based on the width of the pnr-GAL4 expression domain, suggesting that wild-type Yippee protein effects extend somewhat into the pnr > YippeeRNAi stripe: nonautonomous, but short range.

Our data are consistent with Lewis’ hypothesis of sable cell nonautonomy, but might suggest that Yippee + cells can only rescue nearby Yippee− cells within the same tissue. These results are reminiscent of mosaic analysis of another well-known body color gene: In gynandromorphs mosaic for yellow, yellow + cuticle rescued immediately adjacent yellow cuticle (Hannah 1953). Yellow encodes an L-dopachrome isomerase that affects melanin synthesis, this enzyme has a signal peptide that directs transport to the ER, glycosylation and, subsequently, secretion (Drapeau 2003; Hinaux et al. 2018). It therefore makes intuitive sense that yellow exhibits cell nonautonomy. In contrast, the Yippee protein does not have a signal peptide, and so it has been hypothesized to be intracellular (Roxström-Lindquist and Faye 2001). It is possible that Yippee regulates a downstream signal that is secreted or otherwise relayed to other cells; for example, laccase and its sub-strate dopamine are secreted in the melanization pathway, so Yippee might affect this process (True et al. 2005; Yamamoto and Seto 2014; Massey and Wittkopp 2016; Spana et al. 2020). Further investigation of Yippee nonautonomy, such as replication of Lewis’ results using mitotic clonal analysis, seems merited (Germani et al. 2018).

**How the Yippee<sup>able1</sup> mdg4 insertion might affect Yippee function?**

The s<sup>1</sup> strain has an mdg4 retrotransposon element inserted in the 5′-UTR of Yippee (Fig. 5a; GenBank accession # OM135585). Such an insertion could affect Yippee transcript levels and/or structure, and our analysis of Yippee mRNA is consistent with both hypotheses: (1) RT-qPCR showed a roughly 80% reduction of Yippee cDNA levels in s<sup>1</sup> mutants vs s<sup>+</sup> controls (Fig. 5j). (2) All of the s<sup>1</sup> Yippee cDNAs that we cloned and sequenced contained residual mdg4 sequences in the 5′-UTR and were also missing a segment of the 5′-UTR due to splicing (Fig. 6). Most of these altered 5′-UTRs carry upstream ORFs. Any of these mdg4-related alterations could be the cause of the Yippee<sup>able1</sup> phenotype by altering mRNA transcription rate, stability, export, and/or translation rate. The observed temperature sensitivity of Yippee<sup>able1</sup> remains unexplained. One hypothesis is that Yippee<sup>able1</sup> transcript levels and/or splicing may also be temperature sensitive. Alternatively, a downstream or independent element in the pigmentation pathway might itself be temperature sensitive, but it only manifests a phenotype in the presence of reduced Yippee protein levels.

It is likely that at least some Yippee<sup>able1</sup> transcripts were translated into functional Yippee protein, for 2 reasons. First, the observed cDNAs carry a functional Yippee ORF. Second, the Yippee<sup>able1</sup> phenotype is hypomorphic: the phenotype is enhanced over Df(1)Exel245 (Fig. 2) and not as severe as the Yippee<sup>su(s)</sup> and Yippee<sup>A1</sup> strong loss-of-function phenotypes (Fig. 5), suggesting some residual function despite the mutated 5′-UTR and lower transcript level.

The established genetic interaction between Yippee<sup>able1</sup> and suppressor of sable (s<sup>su(s)</sup>) may provide a foothold for further research of Yippee<sup>able1</sup> transcript functionality. Ironically, the mechanism by which suppressor of sable mutations suppress sable is unclear, as molecular studies of s<sup>su(s)</sup> have focused on its genetic interactions with genes that had already been cloned, such as vermilion. s<sup>su(s)</sup> protein is thought to be a component of transcription defense machinery: it binds pre-mRNA that contains TE in-terruption sequence near the 5′ end of the transcript, and there is evidence that this interaction negatively affects transcription rate while targeting transcript for degradation by the exosome. Mutations in s<sup>su(s)</sup> suppress certain TE insertion mutations in the 5′-UTR of other loci by reducing degradation of TE-containing pre-mRNA, allowing for retention, splicing, and nuclear export of more transcript. After splicing and export, it is thought that at least some of the salvaged aberrant mRNA is translated into functional protein, rescuing the phenotype of the suppressed mutation (Searles and Voelker 1986; Rutledge et al. 1988; Searles et al. 1990; Geyer et al. 1991; Murray et al. 1997; Kuan et al. 2004, 2009). Now that Yippee has been identified as the sable gene, the molecular basis of the genetic interaction between s<sup>su(s)</sup> and Yippee<sup>able1</sup> can be investigated.

**CG1673 also affects body color**

As described in our Results, RNAi and mutant alleles of CG1673 lightened cuticle color somewhat (our observations; Mummery-Widmer et al. 2009), IMBA Bristle Screen Database). CG1673 encodes a predicted branch-chain-amino-acid transaminase, which is involved in leucine and valine biosynthesis (Maguire et al. 2015). We did not investigate CG1673 further, but it may be of interest to researchers of body pigmentation.

**Delayed developmental rate**

Yippee<sup>su(s)</sup>, Act5C > YippeeRNAi, and r<sub>4</sub> > YippeeRNAi flies all exhibited delayed development of at least 2 days from egg to adult, without any particular stage clearly presenting as a rate-limiting step. In addition, Yippee<sup>A1</sup> mutants exhibited developmental delay to the pharate adult stage. While not surprising, this phenotype had not been reported for sable mutants before. r<sub>4</sub> is a fat body-specific driver (Lee and Park 2004), Yippee is expressed at high levels in the fat body (Chintapalli et al. 2007; Brown et al. 2014), and r<sub>4</sub> > YippeeRNAi was the only tissue-specific RNAi treatment that we tested that produced a noticeable developmental delay. Therefore, Yippee may act in the fat body to affect developmental rate.

**How Yippee might affect bristles?**

We found evidence that Yippee also affects scutellar, OC, and PV bristle numbers (Table 1). Bristle counts are particularly sensitive to a variety of environmental factors such as teratogens and oxidative stress, making false-positive “mutant” phenotypes
possible (Barik and Mishra 2019; Priyadarsini et al. 2019). However, our negative controls rarely if ever displayed abnormal bristle counts, and each Yippee bristle phenotype was independently verified with multiple genotypes (Table 1): Ectopic scutellar bristles were seen in all 3 Yippee mutant lines, with s-I falling to complement Yippee\textsuperscript{Chi-A} and Yippee\textsuperscript{A1}. A copy of UAS-Yippee rescued s-I ectopic bristles, and ActSC > YippeeRNAi and pnr > YippeeRNAi both phenocopied the mutant trait. In contrast, the missing OC/PV bristle trait did not behave as a straightforward loss-of-function phenotype; among all the genotypes examined for our body color investigation, only s-I/Df(1)Exel6245 and pnr > YippeeRNAi flies were missing OC/PV bristles at significant frequencies. Among the tissue-specific YippeeRNAi treatments that we tested, pnr > YippeeRNAi was the only one that phenocopied the Yippee bristle traits, suggesting that Yippee expression in the ectoderm affects bristle development.

Beyond its apparent ectoderm specificity, how Yippee affects bristle development remains an open question. The bristle sensory organ is produced through multiple rounds of cell division and cell fate determination. At the start of metamorphosis, an array of proneural clusters (PNCs) is established in the developing ectoderm. A single sensory organ precursor (SOP) is selected within each PNC, and this SOP divides asymmetrically to produce pIIa and pIIb. Subsequently, pIIa divides to generate the socket and bristle cells, and pIIb divides to produce the neuron and sheath cells; these 4 descendants of the SOP comprise the bristle sensory organ (Schweisguth et al. 1996; Gomez-Skarmeta et al. 2003; Furman and Bukharina 2008; Schweisguth 2015).

Although disruption of any of the above steps could cause ectopic or missing bristles, the positioning of ectopic bristles may hint at the mechanism underlying the Yippee mutant phenotype. A mutation that causes ectopic PNCs would be capable of producing ectopic bristles that are well-separated from neighboring bristles. On the other hand, a mutation that acts downstream of PNC formation would be expected to produce ectopic bristles that are always adjacent to or adjoining a neighboring bristle, because both bristles originated from the same PNC and/or SOP (Usui et al. 2008; Karbowniczek et al. 2010; Troost et al. 2015; Court et al. 2017). At least to some extent, our analysis of Yippee mutants and YippeeRNAi supported both scenarios: (1) In some cases, ectopic scutellar bristles were well-separated from neighboring bristles (Figs. 3e and 5e; Supplementary Results > Supplementary Fig. S7e). However, (2) the majority of ectopic scutellar bristles were closely associated with a neighbor, even though these bristles always had their own separate socket (Figs. 2c, 2h, 3c, 4d, 5d, 5e, and 5g; Supplementary Results > Supplementary Fig. S7e). (3) Missing OC/PV bristles were only seen in s-I/Df(1)Exel6245 and pnr > YippeeRNAi flies at significant frequencies (Table 1; Fig. 3e; Supplementary Results > Supplementary Fig. S7e). Taken together, Yippee may have some effect on initial PNC formation (1, 3), but more consistently, our observations suggest a role for Yippee at or downstream of SOP formation (2).

Activation of the Notch EGF-like protein by its ligand Delta control several cell fate decisions in the SOP lineage: In the SOP, Notch signaling inhibits adjacent PNC cells from forming ectopic SOPs. Thereafter, Notch specifies pIIa cell fate, then socket and sheath cell fates as pIIa and pIIb divide (Furman and Bukharina 2008; Schweisguth 2015; Sjöqvist and Andersson 2019). Interestingly, some Notch and Delta mutations phenocopied loss of Yippee function. Certain mutations in Notch and Delta cause scutellar bristle duplication or loss, and mutations in the Ahuplex domain of Notch are especially effective at causing loss of OC and PV bristles (Lindsley and Zimm 1992). While Notch itself has not been strongly associated with body pigmentation, several Delta mutations have been associated with dark body color (Schultz 1929; Cramer 1980; Lindsley and Zimm 1992). With these parallels in mind, it is worth considering whether Yippee interacts with Notch/Delta signaling. As discussed above (The Biochemical and Physiological Role of Yippee Remains Unclear), Yippee shares high sequence similarity with YPEL5, a member of the mammalian E3 ubiquitin ligase complex (Hosono et al. 2004; Lampert et al. 2018), and the E3 ubiquitin ligases Neutralized, Mindbomb, and Deltex affect Drosophila bristle formation by regulating the function, endocytosis, and degradation of Notch and Delta (Dietrich and Campos-Ortega 1984; Lindsley and Zimm 1992; Wang and Struhl 2005; Miller and Posakony 2018; Dutta et al. 2021). Taken together, it is possible that Yippee interacts with the Drosophila E3 ubiquitin ligase complex to regulate Notch/Delta signaling in the developing bristle organ.

**How Yippee might affect wing morphology, adult emergence, and cuticle composition?**

The new Yippee alleles, as well as some YippeeRNAi treatments, appeared to disrupt Yippee function more severely than Yippee\textsuperscript{A1}, revealing novel phenotypes such as curved wings and failed adult emergence. Defining tissue/cell specificity is a helpful foothold to investigate gene function. We have this foothold with the Yippee wing phenotype: The nub-GAL4 driver primarily expresses in the prospective wing blade, an ectodermal tissue (Azpiazu and Morata 2000), and nub > YippeeRNAi flies phenocopied the curved wings of ActSC > YippeeRNAi and Yippee\textsuperscript{Chi-A} flies (Figs. 3, h and 5e; Table 1). Therefore, it is likely that wing morphology depends at least in part on Yippee expression within the developing wing ectoderm. The wing is an efficient and sensitive gauge in genetic interaction studies (e.g. Dean et al. 2015; Ellis et al. 2015; Straub et al. 2020), so the Yippee wing phenotype could be a useful starting point to research the mechanisms of Yippee function throughout the fly.

It is unclear whether Yippee affects adult emergence directly or indirectly. Yippee\textsuperscript{A1} pharate adults arrested at P13-14 and did not initiate emergence. In contrast, ActSC > YippeeRNAi and Yippee\textsuperscript{Chi-A} pharate adults at least attempted to emerge, but often became stuck while exiting the operculum. Yippee\textsuperscript{A1} did not show obvious emergence problems. One hypothesis for the difference in severity is that Yippee is a molecular null allele, so it might be expected to cause a more severe phenotype than Yippee\textsuperscript{Chi-A} and Yippee\textsuperscript{A1}, which express some Yippee transcript (Fig. 5). Our experiments do not rule out the possibility that Yippee\textsuperscript{Chi-A} contains a second-site mutation that causes lethality at an earlier stage in addition to the emergence problems seen with the YippeeRNAi and Yippee\textsuperscript{Chi-A} genotypes. Taken together, these observations suggest that Yippee is required for either (1) initiation and/or execution of the emergence behavior or (2) viability at P13-14, shortly before adult emergence can begin. Unlike the body pigment, wing, developmental rate, and bristle phenotypes, the tissue specificity of the Yippee adult emergence phenotype is unknown—indeed, it is possible that this phenotype is not tissue-specific: strong loss of Yippee function might have a generally adverse effect on, for example, metabolism or immunity, leading to lethality at the sensitive transition to the adult stage. Future experiments could test whether Yippee affects adult emergence and viability by its expression in any particular tissue.

Finally, given Yippee’s role in cuticle pigmentation, it may be worth investigating whether it affects other cuticle biomaterial properties. The processes of pigmentation (melanization) and hardening of the adult cuticle (sclerotization) are biochemically
related, both depending on the copper-dependent dopamine monooxidase (laccase) encoded by straw (Suderman et al. 2006; Flaven-Pouchon et al. 2020; Spana et al. 2020). Therefore, it is possible that Yippee regulates sclerotization as well as melanization. Further study could determine if and how exoskeleton composition, crosslinkage, and hardness are affected by Yippee (Jacobs 1985; Flaven-Pouchon et al. 2016, 2020).

Concluding remarks

The authors initially, through our roles as educators and students, undertook this study as a guided inquiry within the classroom. Investigating a 110-year-old question that arose from work by some of the first Drosophila researchers reinforced the relevance of studying classical genetics and, perhaps more importantly, engaged students in the discovery process. The identification of Yippee as the sable gene, its compelling connections to a variety of threads in the literature, and the “Yippee toolkit” provide many opportunities for the research community to investigate a diverse—yet potentially interrelated—array of topics including melanin biosynthesis, the E3 ubiquitin ligase pathway, copper homeostasis, wing development, RNA surveillance, bristle formation, Notch/Delta signaling, and adult emergence.

Data availability

The Supplementary Materials and Methods, Supplementary Data, and Supplementary Results have been deposited on the GSA figshare portal: https://doi.org/10.25387/g3.19059725. The YippeeCh-A and YippeeAl mutant strains are currently available from the authors and have been deposited at the Bloomington Drosophila Stock Center (BL 93858 and BL 93859). Yippee sequences from s1 have been deposited in GenBank under accession number OM135585. The sequences of the YippeeCh-A and YippeeAl mutations are described in the Supplementary Materials and Methods > IV. s1 pupal and adult Yippee cDNA sequences are in the Supplementary Results. Drosophila modular cloning toolkit (Dmo) parts are available from the authors on request. Raw scutal gray value data and P-values for pairwise comparisons (Tukey’s HSD) are provided in an XLSX file (Supplementary Data). Table 1 of this manuscript essentially presents raw bristle and wing data.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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