Supplementary Materials and Methods

I. Fly stocks

The following table lists and describes the fly stocks that were used in this study. Stocks are grouped according to the part of the investigation that they were involved in (**Experiments 1-6**). **(i)-(v)** refer to clarifying footnotes immediately below the table.

TABLE S1. *Drosophila* stock sources, descriptions, and references

Genotype	Source (i) and Description	Refs. (ii)
Experiment 1: Recombination mapping		
s^1	BL 4173. Original *sable*^{1} stock. Used for initial mapping experiments.	1, 2
w wy^2 g^2 *f*	BL 84699. For three-point mapping of *sable*^{1} relative to mapped loci.	3
Experiment 2: Deficiency (Df) and duplication (Dp) mapping (iii)		
w^{1118}	BL 6326. The "s^+" wild-type control stock used for our photos and quantitative data.	4
w^{1118} s^1	The "s^1" stock used for our photos and quantitative data. Made by recombining s^1 from BL 4173 onto the w^{1118} chromosome from BL 6326.	
w^{1118}, Df(1)Exel6245/FM7C *Bar*	BL 7718.	5
w^{1118}; Dp(1;3)DC268	BL 30385.	6
w^{1118}; Dp(1;3)DC269	BL 30386.	6

TABLE S1 (cont.)

Genotype	Source (i) and Description	Refs. (ii)
Experiment 3: Initial screening of *sable* candidate genes (iv)		
w^{1118}; *Act5C*-GAL4/TM6C *Sb Tb*	*Act5C*-GAL4 construct originally from BL 3954, but crossed into a w^{1118} background and balanced over TM6C *Sb Tb*. Ubiquitously expresses GAL4. Also used for experimental crosses in **(4)** and **(5)**.	7, 8
w; pnr-GAL4/TM6B *Tb*	BL 58788 (v). Expresses GAL4 in dorsal ectoderm. Also used for experimental crosses in **(4)**.	9, 10
w^{1118}; *Yippee*^{GD17271}	V 46977. Our primary UAS-*Yippee*RNAi stock. Also used for experimental crosses in **(4)**.	11
w^{1118}; *Yippee*^{GD8633}	V 39899. UAS-*Yippee*RNAi stock. (No longer available from VDRC.)	11
w^{1118}; *CG1673*^{GD9192}	V 25204. UAS-*CG1673*RNAi stock.	11
y^1 w^{67c23} *CG1673*^{EY20842}	BL 23109. Transgene insertion in 5'-UTR of *CG1673*.	12, 13
w^{1118} *CG1673*^{EP1023}	BL 10115. Transgene insertion in 5'-UTR of *CG1673*.	12, 14
Experiment 4: RNAi of *Yippee*		
elav^{C155}	BL 458. Expresses GAL4 in post-mitotic neurons.	15, 16
w; *fkh*-GAL4	BL 78060. Expresses GAL4 in the salivary glands.	17
y^1 w^1; *nub*-GAL4	*nub*-GAL4 construct originally from BL 25754, but then crossed into a y^1 w^1 background (BL 1495). Expresses GAL4 in the wing imaginal disk.	18
y^1 *w;* r4-GAL4	BL 33832. Expresses GAL4 in the fat body.	19

TABLE S1 (cont.)

Genotype	Source (i) and Description	Refs. (ii)
Experiment 5: Misexpression and rescue		
y w; UAS-*Yippee*	F 003701. UAS-*Yippee* provides GAL4-inducible expression of the wild-type *Yippee*-RA isoform with a C-terminal 3xHA tag.	20
w^{1118} s^{1}; UAS-*Yippee*	UAS-*Yippee* construct from F 003701 crossed into our w^{1118} s^{1} background.	
Experiment 6: New *Yippee* alleles, *sable*/*Yippee* complementation tests		
w^{*} $Yippee^{Chi\text{-}A}$/FM7H *Bar*	The $Yippee^{Chi\text{-}A}$ allele has a CRISPaint-induced *mini-*w^{+} insertion immediately 5' of the *Yippee* transcription start site. Constructed as described in this document (**III. Construction of CRISPR mutants**). Deposited at the Bloomington Stock Center (BL 93858).	This study
w^{*} $Yippee^{\Delta 1}$/FM7C *Bar*	$Yippee^{\Delta 1}$ is a molecular null allele, an HDR-CRISPR deletion of the entire *Yippee* coding sequence. Constructed as described in this document (**III. Construction of CRISPR mutants**). Deposited at the Bloomington Stock Center (BL 93859).	This study

(i) Stock providers: Bloomington *Drosophila* Stock Center (BL), Vienna *Drosophila* Resource Center (V), FlyORF (F). Number immediately following a stock center abbreviation is the stock ID.

(ii) Refs. (references) shown in this column: 1. Morgan and Bridges (1916), 2. Lindsley and Zimm (1992), 3. Dean *et al.* (2020), 4. Hoskins *et al.* (2001), 5. Parks *et al.* (2004), 6. Venken *et al.* (2010), 7. Ito *et al.* (1997), 8. Wang *et al.* (2007), 9. Heitzler *et al.* (1996), 10. Calleja *et al.* (2000), 11. Dietzl *et al.* (2007), 12. Bellen *et al.* (2004), 13. Bellen *et al.* (2011), 14. Rorth (1996),

15. Lin and Goodman (1994), 16. Agrawal *et al*. (2005), 17. Henderson and Andrew (2000), 18. Azpiazu and Morata (2000), 19. Lee and Park (2004), 20. Bischof *et al*. (2013).

(iii) **Experiment 2** of this table lists, of all the aberrations that we tested, a deficiency and two duplications that appeared to overlap *sable* (explained in Results). All other deficiencies and duplications that we tested did *not* appear to overlap *sable*; these included: Df(1)ED7170 (BL 8898), Df(1)ED7217 (BL 8952), Df(1)ED7165 (BL 9058), Df(1)BSC713 (BL 26565), Dp(1;3)DC263 (BL 30380), Dp(1;3)DC264 (BL 30381), Dp(1;3)DC265 (BL 30382), Dp(1;3)DC266 (BL30383), Dp(1;3)DC267 (BL 30384), Dp(1;3)DC270 (BL 30387), Dp(1;3)DC272 (BL 30389).

(iv) **Experiment 3** of this table only lists UAS-RNAi lines for the two strongest candidate *sable* genes (*Yippee* and *CG1673*). Other UAS-RNAi lines tested: BL 66975 (*CG1662*), BL 55235 (*Tim9a*), and BL 36717 (*GstT4*).

(v) The *pnr*-GAL4 chromosome of BL 58788 also carries $Moe^{sqh.GFP(S65T)}$, a transgene that expresses an actin-binding motif of Moesin tagged with GFP. $Moe^{sqh.GFP(S65T)}$ does not appear to affect development (Kiehart *et al*., 2000), and we saw no evidence that it significantly affects body color (Figure 3).

II. Experimental crosses

In the preceding section, Table S1 listed this study's fly stocks, subdividing them into **Experiments 1-6**. Here, using the same groupings, we describe how parental stocks were crossed and which progeny were assessed. Most experimental crosses were incubated at 19-21°C (Experiments 1, 2, and 6; *CG1673* crosses in Experiment 3), but crosses involving GAL4-UAS (Experiments 4, 5; GAL4-UAS crosses in Experiment 3) were incubated at 25°C. Cross-referencing this section with Table S1 and with manuscript figure captions should provide the information needed to reconstruct our experimental crosses, but further details can be made available upon request.

Experiment 1: Recombination mapping

- (*w wy*^{2} *g*^{2} *f* females) x (*s*^{1} males), F1 siblings were mated, and F2 males were scored.

Experiment 2: Deficiency (Df) and duplication (Dp) mapping

- Deficiency mapping: (Df(1)/FM7 *Bar* females) x (*s*^{1} males), then F1 *s*^{1}/Df(1) females were scored.
- Duplication mapping: (*s*^{1} females) x (Dp(1;3) males), then F1 *s*^{1}/Y; Dp(1;3)/+ males were scored.

Experiment 3: Initial screening of *sable* candidate genes

- RNAi screen: Deficiency and duplication mapping led to a short list of potential *sable* genes (Figure 2M). UAS-RNAi lines representing most of these candidates were crossed to *pnr*-GAL4 and in some cases *Act5C*-GAL4 flies, and F1 GAL4 > UAS-RNAi flies were examined. Results from this initial screen were cross-referenced with

findings from a previous RNAi-based body color/bristle screen (Mummery-Widmer *et al*. 2009; IMBA Bristle Screen database https://bristlescreen.imba.oeaw.ac.at).

- *CG1673* complementation tests: The $CG1673^{EY20842}$ and $CG1673^{EP1023}$ stocks were crossed to s^1 as well as to Df(1)Exel6245/FM7C *Bar*, then transheterozygous (*CG1673*/s^1) and hemizygous (*CG1673*/Df) F1 females were examined.

Experiment 4: RNAi of *Yippee*

- Experimental groups: w^{1118}; $Yippee^{GD17271}$ females were crossed to males from each of the GAL4 lines listed in Table S1 > Experiments 3 and 4, then F1s carrying a GAL4 and an RNAi construct were scored ("*Act5C* > RNAi", "*pnr* > RNAi", "*nub* > RNAi", *etc*. in Figure 3). (In one exception to this cross scheme, *elav*-GAL4 females were mated to w^{1118}; $Yippee^{GD17271}$ males because the C155 *elav*-GAL4 construct is X-linked, and we wished the GAL4 construct to be inherited by both F1 sexes.)
- GAL4-only controls: w^{1118} s^+ females were mated to males from each GAL4 line, then F1 GAL4/+ heterozygotes were scored ("*Act5C*", "*pnr*", and "*nub*", *etc*. in Figure 3). (In one exception, *elav*-GAL4 females were mated to w^{1118} s^+ males.)
- RNAi-only controls: (w^{1118}; $Yippee^{GD17271}$ females) x (w^{1118} s^+ males), then F1 w^{1118}; $Yippee^{GD17271}$/+ heterozygotes were scored ("RNAi" in Figure 3).

Experiment 5: Misexpression and rescue

- Misexpression, experimental group: (w^{1118} s^+; *Act5C*-GAL4/TM6C *Sb Tb* females) x (*y w*; UAS-*Yippee* males), then F1 w^{1118} s^+/Y; *Act5C*-GAL4/UAS-*Yippee* males were scored ("s^+; *Act5C* > UAS-*Yippee*", Figure 4).
- Misexpression, GAL4-only controls: (w^{1118} s^+; *Act5C*-GAL4/TM6C *Sb Tb* females) x (w^{1118} s^+ males), then F1 w^{1118} s^+/Y; *Act5C*-GAL4/+ males were scored ("s^+; *Act5C*", Figure 4).

- Misexpression, UAS-*Yippee*-only controls: (w^{1118} s^{+} females) x (*y w*; UAS-*Yippee* males), then F1 w^{1118} s^{+}/Y; +/UAS-*Yippee* males were scored ("s^{+}; UAS-*Yippee*", Figure 4).
- Rescue, experimental group: (w^{1118} s^{1}; UAS-*Yippee* females) x (w^{1118}; *Act5C*-GAL4/TM6C *Sb Tb* males), then F1 w^{1118} s^{1}/Y; *Act5C*-GAL4/UAS-*Yippee* males were scored ("s^{1}; *Act5C* > UAS-*Yippee*", Figure 4).
- Rescue, GAL4-only controls: (w^{1118} s^{1} females) x (w^{1118}; *Act5C*-GAL4/TM6C *Sb Tb* males), then F1 w^{1118} s^{1}/Y; *Act5C*-GAL4/+ males were scored ("s^{1}; *Act5C*", Figure 4).
- Rescue, UAS-*Yippee*-only controls: (w^{1118} s^{1} females) x (*y w*; UAS-*Yippee* males), then F1 w^{1118} s^{1}/Y; +/UAS-*Yippee* males were scored ("s^{1}; UAS-*Yippee*", Figure 4).

Experiment 6: New *Yippee* alleles, *sable*/*Yippee* complementation tests

- New alleles: *w* Yippee*$^{Chi\text{-}A}$/Y adult males and *w* Yippee*$^{\Delta 1}$/Y pharate adult males were obtained directly from their balanced stocks (Table S1 > Experiment 6).
- Complementation tests (experimental groups): (*w* Yippee*/FM7 *Bar* females) x (w^{1118} s^{1} males), then F1 w^{1118} s^{1}/*w* Yippee* females were scored ("s^{1}/*Yippee*", Figure 5).
- Complementation tests (controls): (w^{1118} s^{+} females) x (w^{1118} s^{1} males), then F1 w^{1118} s^{1}/w^{1118} s^{+} females were scored ("s^{1}/s^{+}", Figure 5). In a separate set of crosses, (*w* Yippee*/FM7 *Bar* females) x (w^{1118} s^{+} males), then F1 w^{1118} s^{+}/*w* Yippee* females were scored ("s^{+}/*Yippee*", Figure 5).

III. Construction of CRISPR mutants

Plasmid constructs for repair of CRISPR were assembled using MoClo, a modular hierarchical assembly design protocol that uses the Golden Gate reaction to turn standardized parts into complex constructs (Weber *et al.* 2011). We used the connectors and backbones from the MoClo Yeast Toolkit (Lee *et al.* 2015) ('ytk' parts, sourced from addgene.org) and designed *Drosophila* parts, which we refer to with the prefix 'dmo', short for *Drosophila* modular cloning toolkit. Dmo parts are available from the authors on request. We used the Yeast Toolkit's constrained MoClo design rules documented in Lee *et al.* 2015, and we encourage readers interested in the assembly strategy to consult that publication's supplementary text. We switched kanamycin resistance to Level 1 and ampicillin resistance to level 2, and used isoschizomer enzyme Esp3I instead of BsmBI for some reactions. To facilitate reaction setup, we found it helpful to adjust the concentration of each part to 20 nM. A typical 10 µl reaction consisted of 1 µl of T7 ligase, 1 µl of restriction enzyme, either 2x T7 ligase buffer or 10x T4 ligase buffer depending on available reaction volume, 0.5 µl of vector, and 1 µl of each insert part. All enzymes were from New England Biolabs. Reaction cycles were typically 30x(37˚C for 2 min, X˚C for 5 min), 60˚C for 10 min, 80˚C for 10 min, hold at 10˚C, where X= 16˚C for BsaI and Esp3I and 42 ˚C for BsmBI. To incorporate the GFP dropout, we used the end-ligation cycle 30x(37˚C for 2 min, 16˚C for 5 min), 16˚C for 10 min, hold at 16˚C.

MoClo-compatible parts need to be free of BsaI and Esp3I sites, which, as 6-mers, occur frequently in sequences on the scale of *Drosophila* genes. The yeast toolkit specifications also remove NotI sites. We mutated such sites by synonymous

substitutions or permuting base pairs, e.g., CGTCTC to CTGCTC, with PCR primers. PCR fragments were then Golden Gate assembled into MoClo vectors. For example, we built MoClo-compatible dmo-*mini-w* marker genes from the *mini-w* template in the pS3aG vector via PCR mutagenesis and Golden Gate assembly into pEntry. dmo-*mini-w* preserves the intronic XbaI-HindIII deletion characteristic of w^{+mC}, but it ends at the annotated transcription terminus, thus removing the 636bp noncoding sequence that flanks the 3' end of *w* and includes the *wari* insulator (Chetverina *et al.* 2008).

We developed two types of CRISPR experiments to modify the *Yippee* locus, one using CRISPaint (Bosch *et al.* 2020) and one using homology directed repair (Gratz et al. 2014). The CRISPaint strategy (used to make allele $Yippee^{Chi\text{-}A}$) attempts to incorporate a marker construct at a CRISPR cut that is repaired by nonhomologous end joining. Thus, the marker construct is designed to be linearized by a Cas9 cut and does not carry homology arms. A diagram of this CRISPaintL construct is shown in Figure S1.

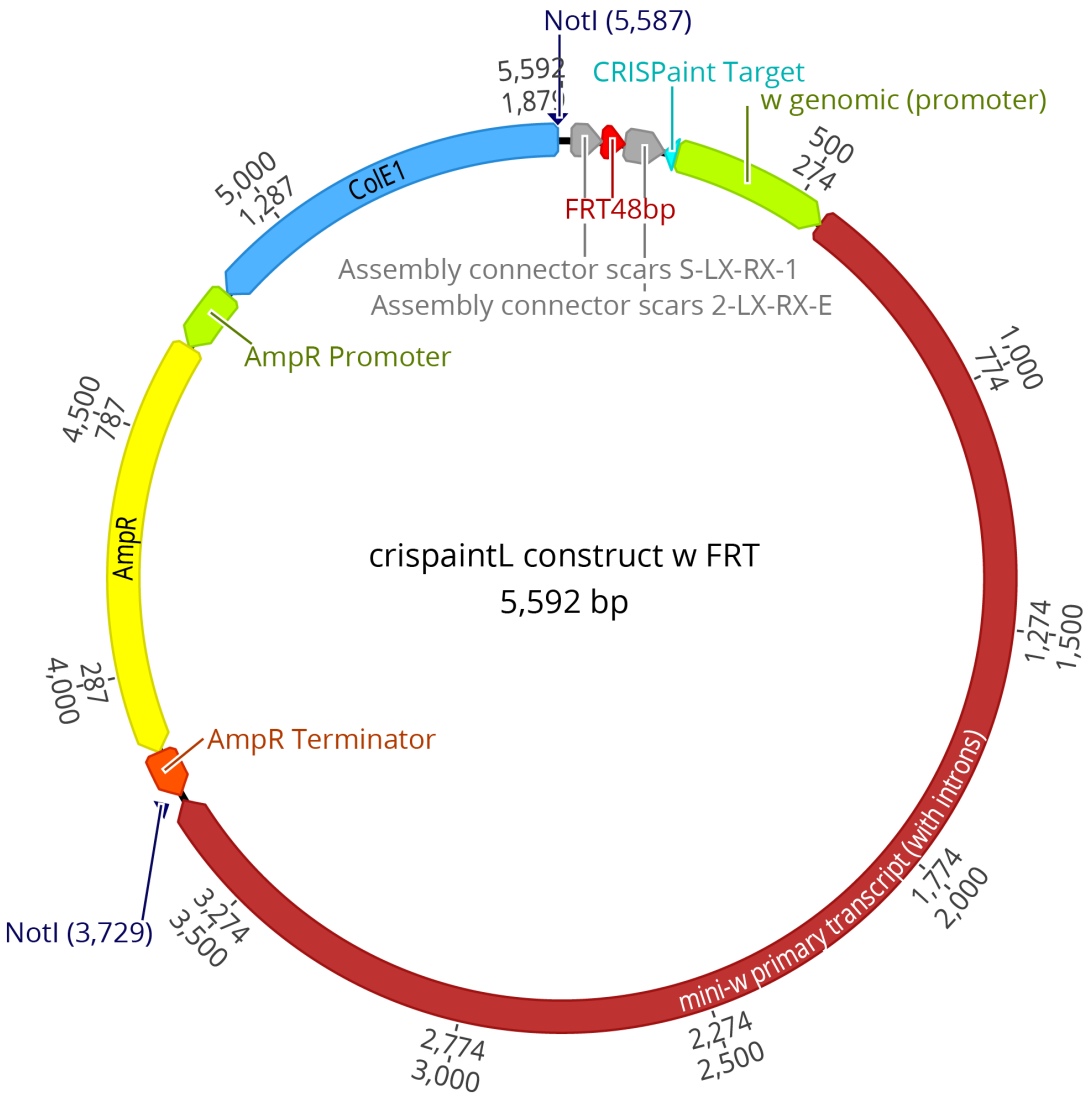

Figure S1. CRISPaint marker construct used to create the *Yippee*$^{Chi\text{-}A}$ allele. This includes the dmo-*mini-w* marker, an AmpRColE1 plasmid backbone, along with some empty MoClo connector slots and a 48bp FRT site (Turan and Bode 2011) that we intended for use in another experiment. The structure is ConS-Con1-FRT48-Con2-ConE-CRISPaint_target - dmo_mini_w-AmpRColE1. Image constructed using Geneious Prime.

The CRISPaintL construct was assembled using MoClo as follows. This Level 2 plasmid was built from four Level 1 parts (t# refers to the part type): a) vector (t1)ConLS'-(t234r)GFPdropout-(t5)ConRE'-(t6)-dmo-CRISPaint_targetL-(t7)dmo-miniw-(t8)AmpRColE1, b) dmo-conS1(empty), c) Con1-(t234)dmo-FRT48-Con2,

and d) dmo-con2E(empty). The 'dmo' parts are newly engineered and we report their sequences here.

- dmo-ConS1(empty) has, along with the Level 1 KanRColE1 backbone, insert sequence
  GCGGCCGCCCCTGAATTCGCATCTAGATGGTAGAGCCACAAACAGCCGGTACAAG
  CAACGATCTCCAGGACCATCTGAATCATGCGCGGATGACACGAACTCACGACGGC
  GATCACAGACATTAACCCACAGTACAGACACTGCGACAACGTGGCAATTCGTCGC
  AATACCGTCTCACTGAACTGGCCGATAATTGCAGACGAACGGCTGCCAATGAGAC
  GTACAGCGGCCGC
- dmo-Con2E(empty) has, along with the Level 1 KanRColE1 backbone, insert sequence
  GCGGCCGCCCCTGAATTCGCATCTAGAATAAATCGCAGCCAAGTGAGTGAATAGA
  TGACGCACCACGGTCAGACACGGCCACATCGTATCTCACAGGAGCAAGCGCGATA
  GGAGCACTCACACATAGTACGGTGATCCGCTGACTCCTTTGCCCAAATAAGACGT
  GAGCCCGTCTCAGATGCACACACTGGCTTAAGATGACAACGGCTGAGCATGAGAC
  GTACAGCGGCCGC
- (t6) dmo-CRISPaint-targetL has, along with the Level 0 CamRColE1 backbone, insert sequence
  AGTCGGTCTCATACACCCCCCGCTTTTTGGGTACTGGCCCGAGTTGAGACCAG
- (t234) dmo-FRT48 has, along with the Level 0 CamRColE1 backbone, insert sequence
  CTGGTCTCACAGCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGG
  AACTTCCGTTTGAGACCGACT
- (t7) dmo-miniw has, along with the Level 0 CamRColE1 backbone, insert sequence
  AGTCGGTCTCAGAGTCTAGTATGTATGTAAGTTAATAAAACCCTTTTTTGCGGA
  AAGTAGATAAAAAAAACATTTTTTTTTTTTACTGCACTGGATATCATTGAACTT
  ATCTGATCAGTTTTAAATTTACTTCGATCCAAGGGTATTTGATGTACCAGGTTCT
  TTCGATTACCTCTCACTCAAAATGACATTCCACTCAAAGTCAGCGCTGTTTGCCT
  CCTTCTCTGTCCACAGAAATATCGCCTGCTCTTTCGCCGCTGCGTCCGCTATCTCT
  TTCGCCACCGTTTGTAGCGTTACGTAGCGTCAATGTCCGCCTTCAGTTGCATTTT
  GTCAGCGGTTTCGTGACGAAGCTCCAAGCGGTTTACGCCATCAATTAAACACAAA
  GTGCTGTGCCAAAACTCCTCTCGCTTCTTATTTTTGTTTGTTTTTTGAGTGATTG
  GGGTGGTGATTGGTTTTGGGTGGGTAAGCAGGGGAAAGTGTGAAAAATCCCGGC
  AATGGGCCAAGAGGATCAGGAGCTATTAATTCGCGGAGGCAGCAAACACCCATC
  TGCCGAGCATCTGAACAATGTGAGTAGTACATGTGCATACATCTTAAGTTCACTT
  GATCTATAGGAACTGCGATTGCAACATCAAATTGTCTGCGGCGTGAGAACTGCG
  ACCCACAAAAATCCCAAACCGCAATTGCACAAACAAATAGTGACACGAAACAGA
  TTATTCTGGTAGCTGTTCTCGCTATATAAGACAATTTTTGAGATCATATCATGAT
  CAAGACATCTAAAGGCATTCATTTTCGACTATATTCTTTTTTACAAAAAATATA

ACAACCAGATATTTTAAGCTGATCCTAGATGCACAAAAAATAAATAAAAGTATA
AACCTACTTCGTAGGATACTTCGGGGTACTTTTTGTTCGGGGTTAGATGAGCATA
ACGCTTGTAGTTGATATTTGAGATCCCCTATCATTGCAGGGTGACAGCGGAGCGG
CTTCGCAGAGCTGCATTAACCAGGGCTTCGGGCAGGCCAAAAACTACGGCACGCT
CCGGCCACCCAGTCCGCCGGAGGACTCCGGTTCAGGGAGCGGCCAACTAGCCGAG
AACCTCACCTATGCCTGGCACAATATGGACATCTTTGGGGCGGTCAATCAGCCGG
GCTCCGGATGGCGGCAGCTGGTCAACCGGACACGCGGACTATTCTGCAACGAGCG
ACACATACCGGCGCCCAGGAAACATTTGCTCAAGAACGGTGAGTTTCTATTCGCA
GTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACT
CAGTTTGCGGCGTGGCCTATCCGGGCGAACTTTTGGCCGTGATGGGCAGTTCCGG
TGCCGGAAAGACGACCCTGCTGAATGCCCTTGCCTTTCGATCGCCGCAGGGCATC
CAAGTATCGCCATCCGGGATGCGACTGCTCAATGGCCAACCTGTGGACGCCAAGG
AGATGCAGGCCAGGTGCGCCTATGTCCAGCAGGATGACCTCTTTATCGGCTCCCT
AACGGCCAGGGAACACCTGATTTTCCAAGCCATGGTGCGGATGCCACGACATCTG
ACCTATCGGCAGCGAGTGGCCCGCGTGGATCAGGTGATCCAGGAGCTTTCGCTCA
GCAAATGTCAGCACACGATCATCGGTGTGCCCGGCAGGGTGAAAGGTCTGTCCGG
CGGAGAAAGGAAGCGTCTGGCATTCGCCTCCGAGGCTCTAACCGATCCGCCGCTT
CTGATCTGCGATGAGCCCACCTCCGGACTGGACTCCTTTACCGCCCACAGCGTCG
TCCAGGTGCTGAAGAAGCTGTCGCAGAAGGGCAAGACCGTCATCCTGACCATTCA
TCAGCCGTCTTCCGAGCTGTTTGAGCTCTTTGACAAGATCCTTCTGATGGCCGAG
GGCAGGGTAGCTTTCTTGGGCACTCCCAGCGAAGCCGTCGACTTCTTTTCCTAGT
GAGTTCGATGTGTTTATTAAGGGTATCTAGTATTACATAACATCTCAACTCCTAT
CCAGCGTGGGTGCCCAGTGTCCTACCAACTACAATCCGGCGGACTTTTACGTACA
GGTGTTGGCCGTTGTGCCCGGACGGGAGATCGAGTCCCGTGATCGGATCGCCAAG
ATATGCGACAATTTTGCCATTAGCAAAGTAGCCCGGGATATGGAGCAGTTGTTG
GCCACCAAAAATCTGGAGAAGCCACTGGAGCAGCCGGAGAATGGGTACACCTACA
AGGCCACCTGGTTCATGCAGTTCCGGGCGGTCCTGTGGCGATCCTGGCTGTCGGT
GCTCAAGGAACCACTCCTCGTAAAAGTGCGACTTATTCAGACAACGGTGAGTGGT
TCCAGTGGAAACAAATGATATAACGCTTACAATTCTTGGAAACAAATTCGCTAG
ATTTTAGATAGAATTGCCTGATTCCACACCCTTCTTAGTTTTTTTCAATGAGATG
TATAGTTTATAGTTTTGCAGAAGATAAATAAATTTCATTTAACTCGCGAATATT
AATGAGATGCGAGTAACATTTTAATTTGCAGATGGTTGCCATCTTGATTGGCCTC
ATCTTTTTGGGCCAACAACTCACGCAAGTGGGTGTGATGAATATCAACGGAGCCA
TCTTCCTCTTCCTGACCAACATGACCTTTCAAAACGTCTTTGCCACGATAAATGT
AAGTCATGTTTAGAATACATTTGCATTTCAATAATTTACTAACTTTCTAATGAA
TCGATTCGATTTAGGTGTTCACCTCAGAGCTGCCAGTTTTTATGAGGGAGGCCCG
AAGTCGACTTTATCGCTGTGACACATACTTTCTGGGCAAAACGATTGCCGAATTG
CCGCTTTTTCTCACAGTGCCACTGGTCTTCACGGCGATTGCCTATCCGATGATCG
GACTGCGGGCCGGAGTGCTGCACTTCTTCAACTGCCTGGCGCTGGTCACTCTGGT
GGCCAATGTGTCAACGTCCTTCGGATATCTAATATCCTGCGCCAGCTCCTCGACC
TCGATGGCGCTGTCTGTGGGTCCGCCGGTTATCATACCATTCCTGCTCTTTGGCG
GCTTCTTCTTGAACTCGGGCTCGGTGCCAGTATACCTCAAATGGTTGTCGTACCT

CTCATGGTTCCGTTACGCCAACGAGGGTCTGCTGATTAACCAATGGGCGGACGTG
GAGCCGGGCGAAATTAGCTGCACATCGTCGAACACCACGTGCCCCAGTTCGGGCA
AGGTCATCCTGGAGACACTTAACTTCTCCGCCGCCGATCTGCCGCTGGACTACGT
GGGTCTGGCCATTCTCATCGTGAGCTTCCGGGTGCTCGCATATCTGGCTCTAAGA
CTTCGGGCCCGACGCAAGGAGTAGCCGACATATATCCGAAATAACTGCTTGTTTT
TTTTTTTTACCATTATTACCATCGTGTTTACTGTTTATTGCCCCCTCAAAAAGCT
AATGTAATTATATTTGTGCCAATAAAAACAAGATATGACCTATAGCCGATGAGA
CCAG

Construction of the repair template for HDR-CRISPR (used to make allele *Yippee*$^{\Delta 1}$) used a new set of vectors we designed to simplify assembly of homology arm constructs. Basically, it consists of a marker segment and a vector backbone segment, carried in separate plasmids (Figure S2), which are assembled together with PCR-amplified homology arms to make a final marker-construct for injection.

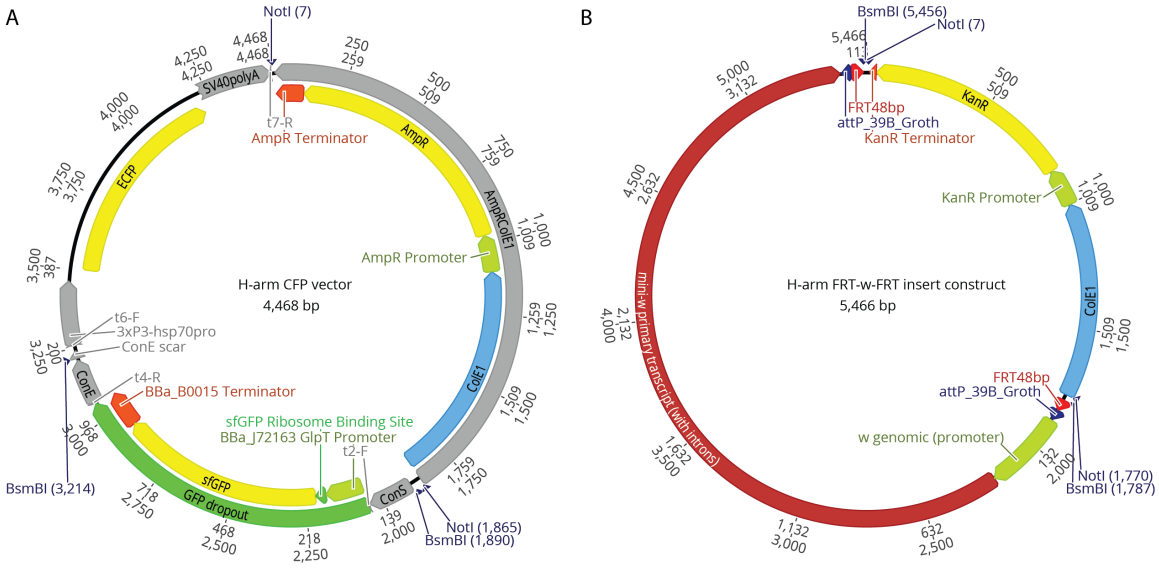

Figure S2. HDR-CRISPR vector and insert plasmids. (A) Vector plasmid "H-arm-CFP" and (B) insert plasmid "H-arm FRT-w-FRT" for making HDR-CRISPR homology arm constructs. The marker plasmid carries *mini-w*, flanked by tandem FRT sites for marker excision and inverted attP sites for recombinase-mediated cassette exchange. The vector backbone is a standard MoClo vector but with a 3xP3-CFP marker to identify unexpected insertion events (an unpublished design idea credited to Kate O'Connor-Giles' lab). Vector and insert bands are purified and Gibson assembled with homology arms. The arms are produced by PCR from primers carrying overhangs that match the MoClo Yeast Toolkit assembly connectors. A revised vector pair that allows assembly by MoClo/Golden Gate as well as Gibson assembly is also available from the authors.

The vector plasmid "H-arm-CFP" has structure (t1)Con1-(t234r)GFPdropout-(t5)Con2-(t67)dmo-3xP3_CFP-(t8)AmpRColE1 (Figure S2). The marker plasmid "H-arm FRT-w-FRT" has structure (t1)FRT48-attP39B-(t234)dmo-mini-w-(t5)attP39Brc-FRT48-(t678)KanRColE1. The minimal attP39B site sequence is from

Groth et al. (2000). The minimal FRT48 site sequence is from Turan and Bode (2011). The 'dmo' parts are newly engineered and we report their sequences here.

- (t1) FRT48 attP39B was amplified from annealed oligos and has sequence GCATGGTCTCACCCTCGTCTCACTGAGAAGTTCCTATTCCGAAGTTCCTATTCTC TAGAAAGTATAGGAACTTCCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGG GGGAACGTGAGACCGCAT
- (t5) attP39Brc FRT48 was amplified from annealed oligos and has sequence GCATGGTCTCAGCTGCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGG AAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGCATGAG ACGTACATGAGACCGCAT
- (t67) dmo-3xP3_CFP was assembled from PCR fragments of 3xP3-CFP that we amplified from gDNA of BDSC strain 32070 to remove BsaI and Esp3I sites. The version used in this paper retains a NotI site in the SV40 terminator. The cloned insert (Level 0, CamR) has sequence AGTCGGTCTCATACAGGATCTAATTCAATTAGAGACTAATTCAATTAGAGCTAA TTCAATTAGGATCCAAGCTTATCGATTTCGAACCCTCGACCGCCGGAGTATAAAT AGAGGCGCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACAC GTCGCTAAGCGAAAGCTAAGCTAATATACAAGCGCAGCTGAACAAGCTAAACAA TCGGGGTACCGCTAGAGTCGACGGTACGATCCACCGGTCGCCACCATGGTGAGCA AGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGA CGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTAC GGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGC CCACCCTCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCGCTACCCCGAC CACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGG AGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAA GTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAG GAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACATCAGCCACAACG TCTATATCACCGCCGACAAGCAGAAGAACGGCATCAAGGCCAACTTCAAGATCCG CCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACC CCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTC CGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTC GTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCC GCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTA AAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTG TTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCA CAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAA ACTCATCAATGTATCTTACCGATGAGACCAG
- (t234) dmo-mini-w has sequence that matches the t7 dmo-mini-w described above but with t2 (AACG) and t4 (GCTG) BsaI overhangs.

For Gibson assembly of the *Yippee* homology arm construct, vector and insert were prepared by 6h enzyme digestion, then the appropriate bands purified by gel extraction. Homology arms were PCR amplified from genomic DNA of the *vasa*-Cas9 expressing strain using primers carrying tails that matched the vector or insert, and then column or gel purified. Vector, insert and homology arms were then assembled using NEBuilder HiFi assembly mix (NEB), cloned, and verified by Sanger sequencing, resulting in the vector shown in Figure S3. The *Yippee* homology arm primers are: yip-Lharm-F CCCTGAATTCGCATCTAGAGTCCAGGCATCCGTTTGTCC, yip-Lharm-R GGAACTTCGGAATAGGAACTTCGGCTGGCGAATAGAGTATTGCAA, yip-Rharm-F TTCTCTAGAAAGTATAGGAACTTCGTATTGAGTAGATGGCCACAGG, yip-Rharm-R GGTTCGTAACATCTCTGTAACCAAAATGGCTAAGACACCGGAAAAC.

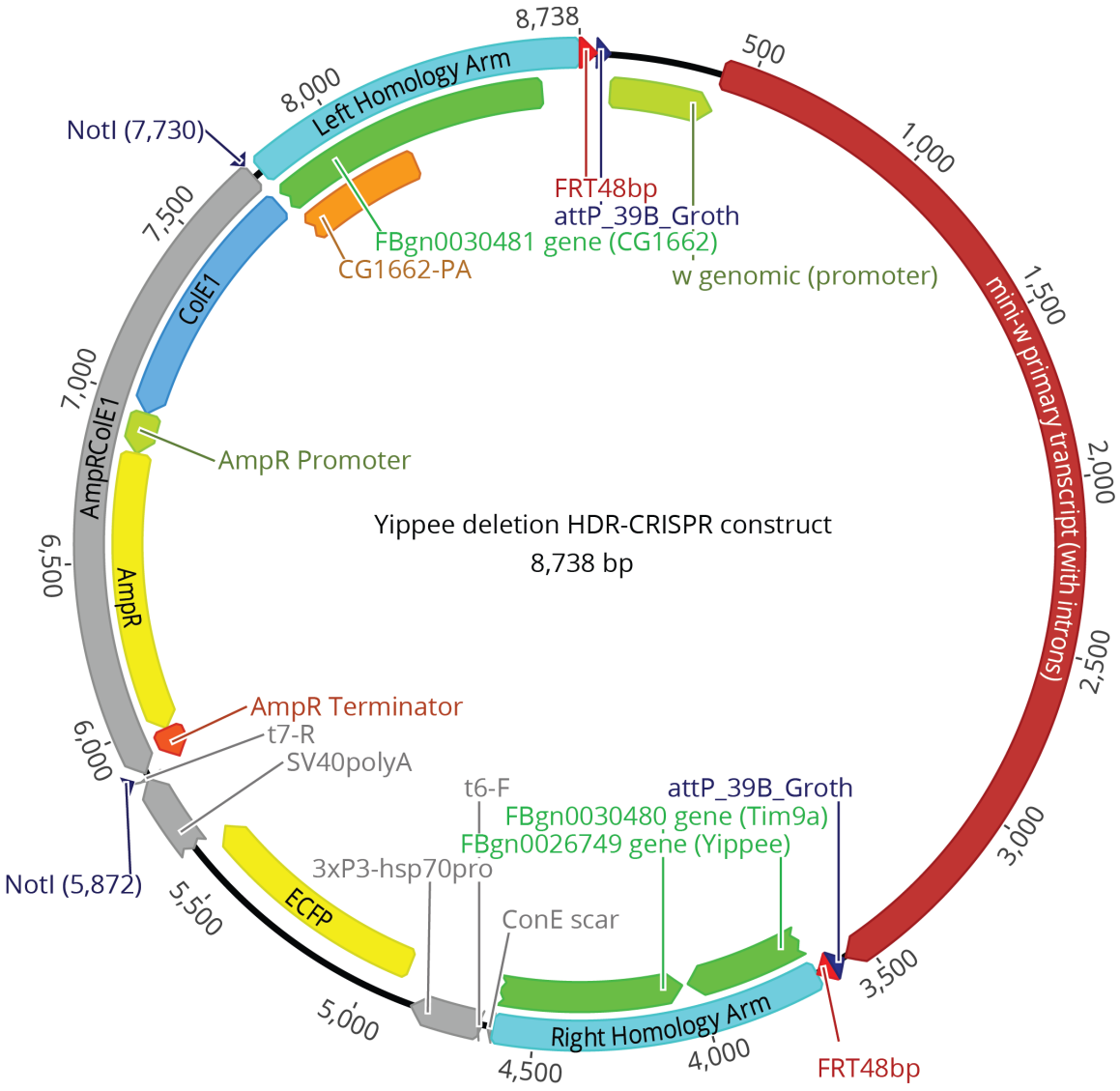

Figure S3. Homology-arm marker construct used to create the *Yippee*$^{\Delta 1}$ allele.

Guide RNAs were chosen from high-scoring predictions of the DRSC Find CRISPRs website (www.flyrnai.org/crispr) and incorporated into pU6-3-chiRNA (Gratz et al. 2014) by the KLD site-directed-mutagenesis procedure (flycrispr.org/protocols/grna). The target sequence of the 5' guide, located in the *Yippee* core promoter region, is gATACAAGTGGACTTAGGGC (with g substituted at the 20th position). The target sequence of the 3' guide, located in the *Yippee* 3'-UTR,

is GGCCATCTACTCAATACTTA. The CRISPaint0 "frame 0 selector" guide, used to cut the donor template, is gccagtacccaaaaagcggg (adapted from Schmid-Burgk et al. 2016; Bosch et al. 2020). After Sanger sequence verification, guide plasmids were prepared for injection using a miniprep kit (Promega), then the eluate was further purified using binding buffer from a PCR cleanup kit and a second miniprep column (Bosch et al. 2020). Marker constructs were purified using the Nucleobond Xtra Midi Plus EF kit (Macherey-Nagel)

Injection mix consisted of guides and repair templates at final concentrations of 50 ng/µl guide and 500 ng/µl repair template. Injections were performed by BestGene, Inc. (Chino Hills, CA) into the vasa-Cas9 strain BL 56552. Red-eyed progeny were initially crossed to *y w* and then the w^+ marker was balanced over FM7.

IV. Determination of structure of new mutant alleles

PCR and Sanger sequencing of junctions between genomic DNA and insert was used to determine the structure of the modification in each fly line. The inferred structure of each insertion is shown in Figure S4.

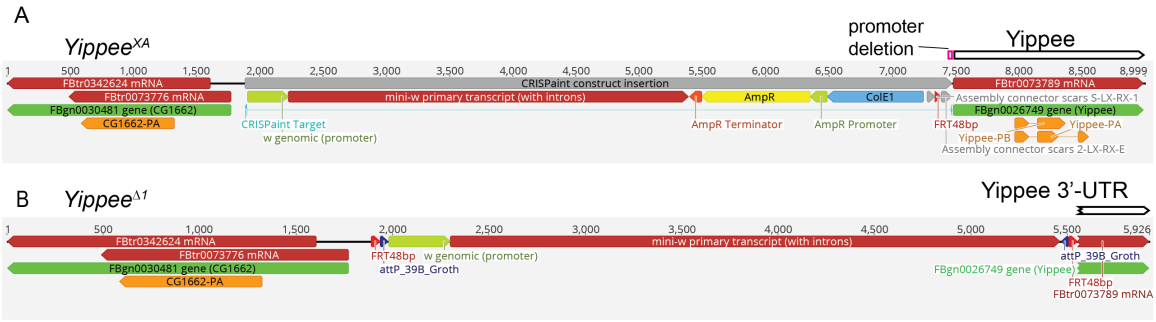

Figure S4. Inferred structure of new mutant alleles. Structure is based on known structure of constructs (Figure S1, S3) as well as PCR and sequences of insertion junctions. Image constructed using Geneious Prime.

For the CRISPaint approach (strain *Yippee*$^{Chi\text{-}A}$), a series of PCRs were conducted to verify the insert location and structure. First, PCRs were conducted using OneTaq polymerase (New England Biolabs) and all combinations of 5'-flanking primers (Yippee-seq-F3 CTGGAGTTAGCTTAGAAAGTTATACAC and Yippee-5'-Region-R1 GTCAGGTGTCCGGTGTCAGGGG), 3'-flanking primers (Yippee-seq-F5 CACAAGACATCTTACTAATGCTCCAA and Yippee-seq-R2 CTCCGTGGCGGATGTGC) and construct-specific primers (S3a-R3 AGTTCAATGATATCCAGTGCAGTA, chk_AmpRColE1-F1 GCTCACATGTTCTTTCCTGCGTTATCCCC). Bands in an agarose gel were observed for primer combinations S3a-R3/Yippee-seq-F3 and chk_AmpRColE1-F1/Yippee-5'-Region-R1, suggesting that the CRISPaint mini-w

construct had inserted only into the 5' site, and that the *mini-w* gene inserted in the same orientation as *Yippee*.

The 3'-UTR site from strain *Yippee*$^{Chi\text{-}A}$ was reamplified using Q5 polymerase then sequenced using primers yippee-seq-F4 CCTGCGTTTCGCAAGTGGAG and yippee-seq-R2 CTCCGTGGCGGATGTGC, yielding a band of the wildtype size, with consensus sequence

TCTGCGACTGCCACCACACAAGACATCTTACTAATGCTCCAATTTATCTCTTAGATACAA
GGAGGGTCGCGTTATCCTGGAGTACGCCCTGATCACAGAGGCAGAGGGCTTTCCGTCGGA
GGCCGCCACCACGAGTCATTGAGCCGATTGAATATACAGCCGGAAAACGGGAACCACGCA
TTGGCGAACCTTGTGTAGCCCT**TATAGGGTA**GAGTAGATGG . This indicates that at this guide RNA target site, the sequence AAGTATT was mutated to TATAGGGTA by repair of the CRISPR cut.

The 5'-UTR site from strain *Yippee*$^{Chi\text{-}A}$ was reamplified using Q5 polymerase and a modified set of primers. The left end was amplified using primers yippee-seq-F3 CTGGAGTTAGCTTAGAAAGTTATACAC and uwseq-R6 GAAAGAACCTGGTACTTCAAATACCCTTGG , resulting in the following consensus sequence (Underline denotes sequence from the inserted *mini-w* construct)

ATACACATTGTTTTTCGAATTTTTTTGCCTGATTTGACTTTTGAAAGGCAATCCGGACAC
ACGCAACAGCAACAACAACAACAATCACGTGTAGGGAACAGCTGTTACGACGAGGGCTA
GTTGGCTTAAGTTATCGATAGTTTAACAATCGTAGCCAATTGAATTTTAAAAAATTTAA
ATAATAAGCCCTTGATATGTTAAAGACAAAAAAGATATCAAAACAACTTAAATTTGTAA
AAAAAAAGTGTAATTTTCAACAAAGAATTTTAAAAGAGACCGATAATGCCTTGCTTGTC
TGGTTGAAAATATTCGTTTGATATATAAACAATAAAATAAAACAGAAACGTAGCACAAA

TGACCAATACTTAATAAATTCAAATATTTTGCAATACTCTATCTCTTTGGGTACTGGCCC
GAGTCTAGTATGTATGTAAGTTAATAAAACCCTTTTTTGCGGAAAGTAGATAAAAAAAA
CATTTTTTTTTTTTTACTGCACTGGATATCATTGAACTTATCTGATCA . The right end of was amplified using primers chk_ampRColE1-F1 GCTCACATGTTCTTTCCTGCGTTATCCCC and yippee-5'-region-R1 GTCAGGTGTCCGGTGTCAGGGG , resulting in sequence CCCTGAATTCGCATCTAGACTGAACTGGCCGATAATTGCAGACGAACGGCTGCCAAACCA
GATGTCAACACAGCTACAACGGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTAT
AGGAACTTCGCTGGATGCACACACTGGCTTAAGATGACAACGGCTGAGCAGTTACAGAG
ATGTTACGAACCACTAGTGCACTGCAGTACACCCCCCTTTTAGCCTTGCATCGATAGCGC
AGAATGCAGTGAC. Thus, TCGCCAGCCCTAAGTCCACTTGTATTCGA was deleted from the *Yippee* promoter region at the 5' guide RNA target site prior to insertion of the *mini-w* construct in strain *Yippee*$^{Chi\text{-}A}$.

For the HDR-CRISPR deletions, six independent insertions (including the *Yippee*$^{\Delta 1}$ line) were checked by PCR using primers in the *mini-w* marker and in flanking genomic sequence not present in the homology arms. All six insertions gave bands of the expected size, suggesting that *Yippee* was correctly deleted and *mini-w* construct inserted in its place, as planned. The left side assay used primers uwseq-R6 and Yippee-Region-F1 GCATCATGCGGCCCCAAACAAACAAGATTAGG, and the right side assay used primers uwseq-F6 ACCATCGTGTTTACTGTTTATTGCCC and Yippee-Region-R1 GAGCACACCCACAAAGCAGGAGC. Sequences were obtained from the *Yippee*$^{\Delta 1}$ PCR products around the junctions of the guide RNA sites; these matched the expected structure with no additional mutations.

V. Photography equipment

Our rig was a variation on the insect photography systems described by Buffington *et al.* (2005) and Kawada and Buffington (2016). Emerged adult flies were visualized through a Zeiss Primo Star compound microscope with standard 10X eyepieces and a Zeiss Plan-ACHROMAT 4X objective lens. (Pharate adults were visualized under a different microscope as described in the next section.) For imaging, a NEX-3N camera body (Sony) was attached to one of the microscope eyepieces with a T-Ring for Sony E Mount and 2-Inch Universal T Adapter (CNC Parts Supply, Inc.). Lighting was provided by two commercial desk lamps pointing at the specimen from either side of the microscope stage, each fitted with a 20W, 120V CFL bulb with 6500K color temperature (General Electric #FLE20HT3/2/D). Although the "light dome" templates provided by Kawada and Buffington (2016) were helpful conceptually, we could not adapt them to accommodate the relative dimensions of our flies and equipment, and so to block direct glare and otherwise diffuse the lighting, a round hole was cut in the middle of a white plastic to-go coffee cup lid, and this makeshift light dome was placed in a skirt-like orientation around the shaft of the objective lens.

VI. Preparation of flies for photography

1-5-day-old adult flies were CO_2-anesthetized, transferred to empty fly vials, and stored in a -20°C freezer. (Age was controlled because *Drosophila* cuticle rapidly darkens immediately after emergence, then slowly darkens thereafter.) In frozen storage, fly tissue dehydrates and darkens very gradually, and this process accelerates after flies are withdrawn from the freezer, so each specimen was photographed within a month after freezing and within about 2 hours after removal from the freezer. For each experiment, experimental and control crosses were set up in parallel. Progeny were collected and frozen concurrently, then photographed within 1-2 days of each other—*i.e.*, before being photographed, experimental groups and controls had been reared under the same incubation conditions, collected at the same adult age range, then stored in the freezer for about the same length of time.

A photography "platform" was prepared by flattening a small lump of white modelling clay onto a microscope slide, then affixing a square of white paper on top of the clay. Flies were placed on the paper platform; body positioning was fine-tuned by gently pressing the platform and underlying clay around each fly.

Yippee$^{\Delta 1}$ mutants died as late pharate adults, failing to emerge from their pupal cases. Rather than freezing these specimens, they were prepared as follows: *Yippee*$^{\Delta 1}$/Y and s^+/Y stage P13-14 pharate adults were dissected out of their pupal cases in 70% ethanol and left in the liquid. Photographs were taken with the same camera and lighting described in the previous section, but using a Zeiss Stemi 305 dissecting microscope.

VII. Photography for assembly of figure images

Figure images were photographed with the rig described in **V. Photography equipment**. Camera settings were ISO 800, 0.5 sec exposure, fine JPEG format, and a 10 second delay self-timer to avoid camera shake. The camera lens had been removed to attach the camera body to the microscope eyepiece; this caused each image to have a shallow depth of field. To obtain figure images with deeper apparent focus, we employed the following focal stacking strategy:

1) The very top of the specimen was placed in focus and photographed.
2) The fine focus knob was adjusted downwards roughly 1.6-2 units, raising the stage about 4-5 μm. Another picture was taken.
3) Step 2 was repeated until all features of interest (dorsal thorax, wings, bristles) had been captured in focus (usually 40-70 exposures).
4) Images were imported into Adobe Photoshop CC 2015. Groups of no more than 10 consecutive images were focal stacked using the Auto-align and Auto-blend tools. Each focal stack overlapped by 4 primary images (*i.e.*, we stacked images 1-10, then separately 7-16, 13-22, *etc.*)—we found that this level of overlap helped blend the stacks seamlessly at Step 5.
5) A series of no more than 10 contiguous stacks were selected that collectively showed all features of interest in focus. In a second round of focal stacking, these stacks were Auto-aligned and Auto-blended into a deeper "meta-stack". This meta-stack was flattened into one image and saved.

Our camera settings had underexposed the images (explained in the next section), so once a set of figure panels had been assembled into a Photoshop file, exposure of the whole file was adjusted +1. One sharpen filter was added to help clarify bristle and wing edges.

VIII. Photography for quantifying body luminance

The following procedure was based on standard photography protocols used in comparative studies of animal coloration (Stevens *et al*. 2007; Bergman and Beehner 2008; De Souza *et al*. 2017; De Souza *et al*. 2020). Using the photography rig described in **V. Photography equipment**, a single photo was taken of each fly with most of its dorsal thorax in focus. Unlike figure images, which were acquired as fine JPEGs, these images were taken in RAW format to avoid loss of pixel information by file compression (Stevens *et al*., 2007). Otherwise, camera settings were identical to those described in the previous section—ISO 800/0.5 sec was an intentional underexposure to prevent loss of gray value data by pixel saturation. For use in white balancing (described in next paragraph), an 18% gray card from the White Balance Card Set (Vello) was imaged with the same photography rig, lighting, and camera settings.

For most experiments, 15 flies/genotype were photographed, but for the misexpression and rescue experiments (Figure 4), 60 flies/genotype were photographed to help discern the relatively subtle differences between s^{+} and s^{1} when flies had been reared at 25°C vs. room temperature. Images were imported into Adobe Photoshop CC 2015. The gray card image was used to establish a white balance levels preset; each fly image was color-corrected with this preset, converted to grayscale, then exported as a TIFF.

IX. Collection and analysis of body luminance data

The TIFF images described at the end of the previous section were opened in ImageJ 1.53i (Schneider *et al*. 2012). Using the Multi-point tool, 20 pixels were randomly selected across a specific area of the scutum of each fly (Figure S5), then the RGB gray values for these pixels were quantified using the Measure tool. [The RGB gray value scale ranges from 0 (black) to 255 (white).] Raw data were transferred to an XLSX file (Supplementary Data). Data were analyzed using JMP and R as described in the Materials and Methods section of the main text.

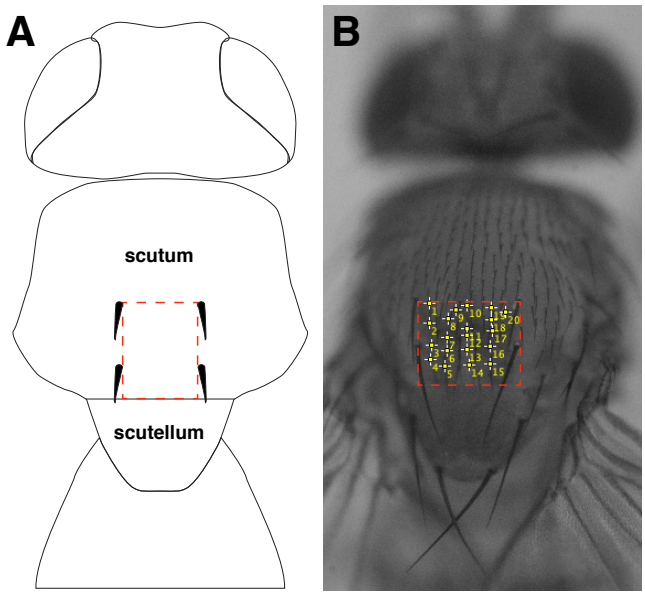

Figure S5. Area of the dorsal thorax where cuticle gray values were measured. **(A)** Schematic of an adult fly, dorsal view. Red rectangle indicates area on the scutum between the dorso-central macrochaetes, extending posteriorly to the scutum-scutellum border. We focused on this portion of the scutum because its pigmentation appeared to be a particularly sensitive indicator of *sable*/*Yippee* function. (Note that this is the region where a "trident" can appear in dark-bodied mutants; example in Figure 1 of manuscript.) **(B)** Example of a fly image in ImageJ, post-analysis. 20 pixels were selected throughout the scutal area of interest (yellow "+" marks), and their RGB gray values were recorded and transferred to an XLSX file (Supplementary Data).

X. References

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